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PREFACE

The reception enjoyed by the first volume of the *Review* has been so cordial that a few words of acknowledgment may not be inappropriate as an introduction to the present volume.

First of all, we feel urged to mention the warm co-operation received from those who have shared in the authorship of the *Review* and to indicate something of the difficulties under which they have labored. Doubtless the most vexing problem has been that presented by the severe limitations imposed upon the length of reviews. The assignments of space were not as generous as we would have wished and the authors frequently found themselves obliged to eliminate ruthlessly paragraph after paragraph of manuscript. To the reviewer there are only two solutions to such a problem, neither of which is ideal. Either a superficial and hasty mention may be made of most of the papers appearing within the period under review, or a portion may be selected for more exhaustive and critical analysis. Although reviews of the former type are of obvious value for bibliographic purposes, we have come to believe that surveys of a less comprehensive but more critical nature will be of greater usefulness in this *Review*.

Indeed, so fertile are the fields of biochemistry that the preparation of complete and comprehensive surveys of the rapidly growing literature would tax beyond measure the ingenuity and industry of the chronicler. New journals are being born over night and little if any abatement is yet evident in the flood of papers to the old. On vitamins alone no less than 1,000 papers are reported to have been published in the past year. In the present volume almost 3,000 papers in twenty-five different fields have actually received review. We are satisfied that this represents less than half of the papers of sufficient merit and weight to deserve treatment.

The selection of the papers has presented numerous difficulties to the reviewers. Many were put aside because they dealt with isolated topics which cannot yet be profitably reviewed. Others pertained to questions of major importance and interest but, because of the exigencies of space, were deliberately reserved for treatment in the next or a later volume. Still others may have been inadvertently overlooked.

The task of the reviewers has been somewhat lightened by the

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cordial co-operation of the many who have been good enough to send reprints of recent publications. These have proved of special value, particularly in the case of works appearing in journals not readily accessible to the reviewer.

Others have displayed their interest by suggesting ways in which the usefulness of the *Review* might be further enhanced through changes or additions in content. The growth of a subject is characterized by its fluidity. Activity in biochemical research shifts from field to field, and interest waxes and wanes. Such transitions in emphasis require recognition. From time to time certain sections must be expanded and others reduced. New topics deserving of review must be included in replacement of the old. The Committee will continue to welcome most warmly all suggestions bearing on these and kindred matters.

Again we wish to acknowledge gratefully the support which the *Review* enjoys from the Chemical Foundation. Without its endorsement and financial aid, inception of the enterprise would have presented the most serious difficulties.

To the Stanford University Press our thanks are due for their patience and care in composition, and for the excellence of their workmanship.

It is the hope of all connected with the *Review*—the authors, the editor, the advisory committee, and the Press—that the present volume may deserve no less fully the generous support received by that of last year.

J. M. L.
C. L. A.
D. R. H.
C. L. A. S.

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PERMEABILITY*

By RUDOLF HÖBER

University of Kiel, Germany

Following the plan of a year ago¹ this review upon the experimental work of 1932 deals with the passage of water or dissolved substances into and through cells or tissues. To what extent may this be considered as a spontaneous process, comparable to diffusion and osmosis, and to what extent must it be regarded as dependent on energy expenditure? From this point of view we shall concern ourselves first with cell permeability and secondly with tissue permeability.

I. CELL PERMEABILITY

In plant cells, very often, permeability has been studied by plasmolysis. But many investigations have called attention to different circumstances, which may discredit the method in various cases. Thus it has been pointed out by Weber in several experimental contributions that plasmolysis often increases the permeability to such a degree that the cells are killed by the penetration of the plasmolyzing substance. With cells of *Helodea canadensis* the injury is augmented by cooling to 1°–3° C. It can be assumed that the injury consists in the tearing of the protoplasmic surface and that the reparation, normally accomplished spontaneously, proceeds too slowly. Another explanation for the injurious effect of plasmolysis at low temperatures is based upon the observation that the alteration of the protoplasmic surface generally is greater, since the plasmolysis is brought about with greater difficulty; and since low temperatures increase viscosity and adhesion of the protoplasm, it is to be expected that the injury would be greater. Opposed to the effect of cold is that of narcosis, in which the viscosity decreases. Accordingly, the increase in permeability and cell injury have been shown to be prevented by 0.5 per cent chloralhydrate or 1 per cent ether. If, before plasmolyzing with 2 M urea, *Spirogyra* be exposed for one minute to 1 M oxalate, the cells die; by addition of CaCl₂ to the urea solution the cells can be protected, apparently because the presence

* Received January 10, 1933.

¹ See Höber, R., *Ann. Rev. Biochem.*, 1, 1 (1932).

of calcium is necessary for the recovery of the damaged plasma membranes.

A paper by Hoffmann deals with the determination of the osmotic pressure in marine algae by plasmolysis. This determination is often rendered difficult by the swelling of the cell membrane to the inside of the cell as soon as the osmotic pressure of the plasmolyzing solution surpasses the turgor, so that a real plasmolysis cannot appear. Plasmolysis does not become visible before the swelling has reached its maximum. Therefore, according to Hoffmann, it is better to replace the method of plasmolysis by determining, under microscopic observation, the concentration at which the cell membrane begins to thicken. The observed concentrations agree sufficiently well with the liminal concentrations for shortening the cells along the longitudinal axis by osmotic forces in excess of the cell-turgor. Special influences on the swelling of the membrane dependent upon the chemical composition of the medium can be avoided best by the use of more or less concentrated sea water as plasmolyticum. Comparing the liminal concentrations of different solutes for shortening the cells, it appears that some species of marine algae like *Elachista* and *Chaetomorpha* are still permeable for glucose—even for sucrose. Finally, the experiences of Hoffmann agree with those of Weber, previously referred to, that removal of the protoplast from contact with the cell membrane by plasmolysis often destroys the surface of the protoplasm.

Observations of Lucké concern the osmotic behavior of living cell fragments. They supplement former experiments of McCutcheon, Lucké, and Hartline with intact cells, by which it has been pointed out that in unfertilized eggs of the sea urchin *Arbacia* a remarkable part of the volume (about 11 per cent) is occupied by osmotically inert material ("*nicht lösender Raum*"). This is analogous to well-known observations of Hamburger, Ege, and others on erythrocytes. Lucké inquires as to whether or not this inert material is distributed regularly throughout the cell body. To answer the question he employs a method of E. N. Harvey whereby the eggs are separated into two nearly equal spheres by means of a strong centrifugal force. One of the spheres is pigmented and contains the great bulk of the yolk and pigment granules; the other is colorless and contains the nucleus, fat, and a small amount of the yolk. Both fragments, as well as intact eggs, are distributed in sea water and measured. Subsequently they are brought into 60 and 70 per cent sea water and measured again. The graphic analysis of the swelling curves shows

that nearly twice as much osmotically inactive material is contained in the pigmented spheres as in the colorless spheres. The fragments of the eggs can be regarded as living because they form a fertilization membrane and cleave. But the swollen cells, returned to ordinary sea water, no longer shrink as completely as the whole cells; apparently the normal semipermeability has been altered to some degree.

Permeability to solutes.—The method by which Huber and Höfler, and McCutcheon, Lucké, and Hartline have tried to get an exact measure for the rate of penetration of water through the surface of cells has already been outlined.² In a similar way, Jacobs and Stewart now endeavor to define cell permeability to dissolved substances as the amount of substance entering the cell through unit surface in unit time with unit difference of concentration between inside and outside. The problem is difficult because the penetrating solvent and the penetrating solute influence each other's behavior in a complicated manner. For example, if we have to do with an osmotic disturbance which has been brought about in cells like those of the egg of the sea urchin *Arbacia* by increasing the osmotic pressure of the sea water through the addition of a penetrating, but indifferent, organic substance, the entrance of the dissolved substance S is given by the equation

$$\frac{dS}{dt} = k_1 A \left(C_s - \frac{S}{V} \right) \quad (1)$$

where k_1 is the permeability constant for the substance S ; A is the area of the cell surface; C_s represents the external concentration of S , V the volume of the cell, and $\frac{S}{V}$ the internal concentration.

Further, remembering that the volume changes that occur are determined by the amount of water which crosses the surface, we have

$$\frac{dV}{dt} = k_2 A \left(\frac{a + S}{V} - C_m - C_s \right) \quad (2)$$

where a is the amount of osmotically active materials originally present in the egg, S is the amount of penetrating substance which has entered, C_s the concentration in the external medium, and C_m the

² See *Ann. Rev. Biochem.*, 1, 10 et seq. (1932).

original concentration of the external medium. If we rearrange Equation 2 into another more practical form, we have

$$S = \frac{V}{k_2 A} \cdot \frac{dV}{dt} - a + (C_m + C_s)V \quad (3)$$

Now since the egg in undergoing shrinkage followed by swelling passes through a minimum volume, the value of $\frac{dV}{dt}$ for this volume is equal to zero, and Equation 3 simplifies to

$$S = -a + (C_m + C_s)V \quad (4)$$

where S is the amount of substance which has entered the cell up to the attainment of the minimum volume.

Further, the decrease of V and A being so small in the experiments—it is of the order of magnitude of 10 per cent—that their value can be assumed as constant, Equation 1 can be integrated, giving

$$k_1 = \frac{V}{At} \cdot \ln \frac{CV}{CV - S} \quad (5)$$

It appears that k_1 can then be evaluated from Equations 4 and 5.

The application of the method is demonstrated in a series of experiments with unfertilized eggs of *Arbacia*. The concentration, C_m , of the sea water corresponds with a 0.95 molar solution of some non-electrolyte, $C_s = 0.5$, $a = 0.95 V$. The value of k_1 is calculated in such a way that it indicates the number of mols that would enter the cell through 1 sq. μ of surface in one minute with a concentration difference between inside and outside of 1 mol per litre. The permeability constant $k_1 \cdot 10^{15}$, evaluated in this way for five non-electrolytes, increases in the series: glycerol (0.5), ethylene glycol (3.6), acetamide (5.75), propionamide (14.2), butyramide (36.6).

In a second paper the procedure just described has been applied by Stewart and Jacobs to the following problem: Lillie and others have demonstrated that the fertilization of the sea urchin egg is followed by an increased permeability to water. It was desirable to extend the studies to the dissolved substances. The volume of *Arbacia* eggs was estimated by the technique of McCutcheon and Lucké; thereafter the eggs were exposed to a 0.5 molar solution of ethylene glycol in sea water, and the ensuing shrinking to the minimum value

and subsequent swelling to the initial volume were observed. The shrinkage reached its maximum in from two and one-half to three minutes in unfertilized and in from three-fourths of a minute to two minutes in fertilized eggs. The swelling to the initial volume was accomplished in about twenty minutes in unfertilized and in from ten to fifteen minutes in fertilized eggs. The calculation of the permeability constant, $k_1 \cdot 10^{16}$, according to the aforementioned formulae, gave the average value of 3.6 for unfertilized and of 9.8 for fertilized eggs. In addition to these experiments, some estimations were made on eggs with artificial membranes, the formation of which had been induced by a short exposure of the eggs to distilled water; the alteration increased the permeability constant to an average value of 7.4. The highest value, 10.2, was obtained with unfertilized, but mature, starfish eggs; their permeability underwent little change on fertilization.

A preliminary communication of Thörnblom concerns the same problem of the rate of penetration of dissolved substances in the fertilized and unfertilized eggs of the sea urchin. A number of non-electrolytes—urea, thiourea, dimethylurea, diethylurea, and ethylene glycol—were dissolved in sea water, and the shrinkage and subsequent swelling of the eggs in the hypertonic medium followed. The complicating influence of the permeability for water on the quantitative measurement of penetration of the dissolved substance was eliminated here in the following way. The decrease in volume was observed after the addition of a non-penetrating substance like glucose; in this case the decrease to a minimum is not succeeded by an increase, as in the first case. By comparing the two curves of volume change, it was possible, by use of the second, to introduce a correction into the first for the permeability for water. In this way it was found that the permeability for solutes is increased by fertilization. The first cleavage has a much smaller influence and relates especially to the permeability for water. The penetration of dimethylurea and diethylurea exceeds by far the penetration of urea and thiourea.

Corresponding observations have been published by Hobson.

Permeability to ions.—Jacobs and Parpart call attention to the fact that it is customary to suppose that the erythrocytes are readily permeable to hydrogen ions, though they are well known to be permeable to anions and in general impermeable to cations. In this respect the behavior of the hydrogen ion would represent a single exception to a general rule and would require another and more com-

plicated theory of ionic permeability. Therefore it seemed necessary to the authors to determine whether the supposed permeability to hydrogen ions was not, in reality, a permeability to hydroxyl ions. For studying the entrance of acid into the erythrocytes the authors made use of hemoglobin as an indicator, which in the presence of sufficiently high concentrations of acid loses its red color. Usually the color change is accompanied by hemolysis, but by various methods the color change could be caused to precede, more or less, the hemolysis. Therefore the authors believe that in general the acid first acts upon the hemoglobin before it destroys the cell. If to erythrocytes distributed in 0.3 M sucrose + 0.02 M HCl there are added different amounts of NaCl, the time of color change and the rate of hemolysis are greatly accelerated. This phenomenon is not to be explained by an increase of permeability similar to the increase produced by sodium salt in certain experiments on plant cells, and it is not due to the removal of some important substances from the cell surface by the salt. As experiments show, the probable explanation is as follows: in salt solution the rate of penetration of acid is only slightly affected by its concentration, while in 0.3 M sucrose it is approximately proportional to its concentration. This disagrees completely with the rates of penetration of hydrogen ions, which can be roughly calculated from the mass law, because the calculation postulates for the NaCl solution an increase in rate somewhat greater than the rate of increase in hydrogen ion concentration, and for the sucrose solution an increase in rate approximately proportional to the square of the hydrogen ion concentration. On the other hand, the experimental results harmonize sufficiently well with the hypothesis of permeability to hydroxyl ions, in so far as the calculation predicts that in the salt solution the concentration of acid is of small importance, whereas in the non-electrolyte solution the rate of color change and hemolysis are proportional to the concentration of acid. Further, it has been observed that in sugar solutions, at acid concentrations which readily bring about the color change in the presence of NaCl, this change fails entirely, which is likewise in agreement with the theory of permeability to the hydroxyl ion.

In a previous paper Sen has shown that the stimulation of plant tissue mechanically or by an induction shock is followed by a decrease of the electrical resistance. In a later paper the author demonstrates that the decrease of resistance is accompanied by an exosmosis of ions. In his experiments a *Nitella* filament was placed on a slide

in a thin film of water; electrodes of fine platinum wire were fixed to the ends of the filament; and with a micromanipulator micro-electrodes were brought into the water at a distance of 10μ from the cell surface. The cells were then stimulated by single induction shocks. Only when the shock was strong enough to stop the streaming movement of the protoplasm was there a deflection of the galvanometer attached to the micro-electrodes, indicating an exit of ions. For minimal stimuli a decrease of about 20 per cent in the resistance of the film of water was observed. Weak stimulation was followed by recovery; the resistance of the water again increased and the protoplasmic streaming returned. Stronger stimulation killed the cells; the fall of resistance was permanent.

Michaelis has pointed out that the collodion membrane is increasingly permeable to alkali ions in the order of decreasing ionic radii $\text{Li} < \text{Na} < \text{K} < \text{Cs}$. The protoplasmic membrane seems to behave in a quite different manner, for several authors have shown that *Valonia* is practically impermeable to Cs. In the opinion of Brooks the reason may be that some of the alkali ions alter the protoplasmic surface in a special way; e.g., Scarth has found that the permeability of *Spirogyra* is increased by Li and decreased by Cs. Therefore Brooks has extended the investigations on the properties of the alkali ions by experiments with Rb. Large cells of *Valonia* were distributed into isosmotic RbCl solution diluted by sea water; the concentrations of RbCl made up in this way were about 0.006 to 0.024 M. After from one to nine days the sap was extracted from the cells and Rb determined gravimetrically as Rb_2SnCl_6 . It was found that Rb accumulated in the cell sap to a remarkably high degree, comparable to that of K. But the average rate of intake of Rb was about 20 times as great as that of K and 120 times as great as that of Na. This behavior suggests that *Valonia* is more permeable to Rb than to K, so that the speed of penetration follows the order of decreasing ionic radii, $\text{Na} < \text{K} < \text{Rb}$, as is also the case for dried collodion membranes. As far as Li and Cs are concerned, it seems probable that their apparently anomalous positions are due to secondary influences on the permeability of the cells.

Intake and accumulation of other ions in plant cells have been studied by Steward. In relation to the well-known experiments of Hoagland and Davis on the absorption of salts by *Nitella*, Steward investigated the absorption of KBr by storage tissue of higher plants, using potato discs. It appears that some experimental conditions,

often neglected by other workers, were of decisive importance. Especially, the great oxygen consumption and carbon-dioxide production of the potato must be taken into account. It was shown that the intake of salt depended markedly on aeration with carbon-dioxide-free air because of the high gaseous metabolism of the tissue. Furthermore, the absorption of salt decreased with temperature and ceased below 5°–6° C. Mechanical agitation disturbed the process also. Light had no influence. Under good conditions it appeared that the external solution lost potassium and bromide ions during more than one hundred hours, and that during this time the ions accumulated to a high degree in the readily expressed sap, where they existed in true solution. It seems that the cation and the anion are absorbed in equivalent amounts. The accumulation ratio attained in a given time by a given amount of tissue became the greater, the more dilute the external solution. Under the best conditions the accumulation ratio exceeded one thousand. It must be concluded that the accumulation is dependent upon the performance of work by living cells, at the expense of energy whose source is given in the respiratory metabolism of the carbohydrate depots of the storage tissue.

The same problem of osmotic work and its thermodynamic basis has been discussed during the last three years in connection with the assertion of Straub that the yolk membrane of the hen's egg, in spite of being permeable for water, maintains an osmotic pressure difference between yolk and white of nearly 2 atmospheres, calculated from the difference of freezing-points of about 0.16° C (0.60° — 0.44°). The experiments of Straub have been repeated and varied in several directions (chemical studies, vapor-pressure determinations, dialysis experiments) by a number of authors without fully satisfying results. A further study of the question has been reported by Howard. New determinations of the freezing-point showed that there is no difficulty in obtaining a constant freezing-point for egg white, but that the yolk was inclined to give inconstant and deceptive values below the freezing-point of the white, if the technique were faulty (too great supercooling, insufficient stirring); for it must be borne in mind that the high viscosity of the yolk, the low water content, the high fat content, and the remarkably slow rate of freezing, as the freezing-point is approached, cause anomalous behavior. With improved technique the freezing-points of white and yolk were shown to be identical. Further experiments pertain to the question

as to whether the yolk and a salt solution, which is allowed to come into equilibrium with the yolk across a collodion membrane, have the same freezing-point. Here, experimental difficulties were shown to arise from the passage into the dialysate of a substance present in the yolk which inhibits the crystallization of ice. A satisfying explanation of the phenomenon is lacking. Finally, a series of vapor-pressure determinations were made by the dynamic method of Washburn and Heuse, with the result that the freezing-point, as calculated from the vapor pressure, was in satisfactory agreement with the value obtained directly. Thus Howard comes to the conclusion that there is no osmotic pressure difference between the yolk and the white of the hen's egg; yolk and white are in osmotic equilibrium.

A new contribution to the much discussed question of experimental change in permeability has been offered by Lepeschkin. The first series of experiments deals with the influence of narcotics upon the penetration of aniline dyestuffs into the plant cells. Leaves of *Helodea*, cut from the same branch, were put in dye solutions containing a series of concentrations of chloroform or ether, and two hours later the intensity of staining was determined colorimetrically by a method described in a former paper. Preliminary experiments had indicated that the adsorption of the dyes on the cell wall, as well as the absorption by the cell sap, could be neglected in comparison with the staining intensity of the protoplasm. Three dyestuffs were used in 0.0008 per cent concentration: methylene blue, neutral red, and gentian violet. It was found that the permeability of the protoplasm for the dyes is regulated by their relative solubility in water and chloroform or in water and ether. Methylene blue is much more soluble in water than in chloroform and is insoluble in ether. Neutral red is much more soluble in chloroform, as well as in ether, than in water. Gentian violet is much more soluble in chloroform than in water and is insoluble in ether. In accordance with these differences, the coloration during narcosis decreased with methylene blue in both cases and increased with neutral red; but with gentian violet the coloration was diminished by ether and raised by chloroform. Only in strong solutions of narcotics, which are poisonous to the cells, was the destructive effect in any case accompanied by increased coloration.

Experiments communicated in the second part of the paper deal with mechanical agents which produce an increase of permeability in *Helodea* leaves or in epidermis of *Tradescantia*. The agents are

plasmolysis and deplasmolysis, cutting and bending. The increase of permeability was demonstrated either by coloration with methylene blue or by the method of isotonic coefficients. It was observed that, immediately after cutting, the cells adjacent to the injured part were much more permeable than before and than some hours later, after recovery had taken place. Furthermore, the cells were protected against the injurious effect by a sufficient quantity of chloroform.

In a third series of experiments it was pointed out that the permeability is increased by illumination, but that narcotics are unable to prevent this effect.

II. PERMEABILITY OF TISSUES

Liver.—Höber and Titajew have observed that the isolated liver of the frog, perfused with Ringer solution, is able to secrete almost any aniline dyestuff added to the perfusion fluid in a very low percentage (0.0005 per cent). The performance of osmotic work in this process by the living gland is indicated by the fact that the concentration of the dye in the secretion surpasses in many cases a thousand times that of the perfusing solution. According to Höber this work of the gland can be more or less stopped, and reversibly so, not only by narcosis or by treatment with cyanide, but also by the addition of indifferent, surface-inactive, lipoid-insoluble non-electrolytes in relatively small concentration (about $\frac{1}{8}$ isotonic). This effect is generally greater with increase in molecular size of the non-electrolyte. For example, the inhibition was strong with disaccharides, hexitols, and hexoses; a little weaker with pentoses; still weaker with thiourea, malonamide, lactamide, and glycerol; and lacking with acetamide and urea. Aminoacids, like alanine and glycine, were more effective than would be expected, because their molecular volume in solution is increased by hydration. The pronounced relation between cessation of the effect and molecular volume suggests that the secretory function of the gland has something to do with the penetration of the dyes through any pores the diameters of which are changed in some way by the non-electrolytes. The sodium salts of a number of aliphatic acids resembled the non-electrolytes. The secretion of the dyestuffs by the kidney of the frog was not inhibited in a comparable manner by the non-electrolytes.

Salivary gland.—In regard to the well-known observations that saliva is highly hypotonic to the blood, that its ion content is small, that the percentage of potassium in most cases considerably exceeds

that of serum (while the behavior of sodium is opposite), and that several organic substances dissolved in the blood do not appear in the secretion, a question arises as to the nature of the laws that govern the permeability of the salivary gland. A satisfactory answer is afforded by an experimental procedure in which the concentrations of dissolved substances in the perfusing fluid can be compared exactly with those in the secretion, i.e., a technical arrangement by which the isolated surviving gland can be perfused with a single artificial medium.

In experiments of Amberson and Höber a preparation of the submaxillary gland of the cat is described which is able to produce for one hour or longer after stimulation of its nerves a secretion the composition of which indicates an approximately normal permeability. The perfusion medium of the isolated organ is warm oxygenated Ringer solution to which hemolyzed ox-blood corpuscles have been added.

Up to the present the investigations have been concerned with the permeability of the gland for a group of organic non-electrolytes of relatively low molecular size. The results have been as follows. When a number of acid amides were added to the perfusion fluid, correlations were revealed between their ability to penetrate into the saliva and both their lipoid solubility and molecular volume. Lipoid-soluble amides, like butyramide, as well as those of low molecular volume, like acetamide and propionamide, appeared in high concentration in the saliva, whereas those which are lipoid-insoluble and of greater molecular volume, like malonamide and asparagine, showed little penetration. Glycine and alanine did not pass through the gland. Their real molecular volumes, in aqueous solution, appear to be much greater than those evaluated from their molecular refractions. This suggests that their dissolved molecules are enlarged by water-dipoles. The gland is only slightly permeable for glucose if at all. The smaller-sized dihydroxyacetone penetrated with relative ease. An interesting exception is afforded by urea and monomethylurea, in so far as they penetrated less readily than was to be expected from their small molecular volumes. The lipoid-soluble dimethylurea went through with ease.

Muscle.—Achelis has studied the permeability of frog's muscle to ions by measuring the capacity of polarization. Like Gildemeister, he made use of a Wheatstone bridge arrangement with sinusoidally alternating current of from 400 to 15,000 cycles. Three of the four

arms of the bridge are induction-free; the fourth contains the organ and a variable inductance which compensates for the shift of phase brought about by the polarization of the organ. Increasing resistance and increasing inductance are indicative of increased capacity of polarization or decreased permeability, probably because of a condensation of the membrane material. On the other hand, decrease of resistance and inductance signifies increased permeability or loosening of the membranes.

In this way Achelis has measured the permeability of the abdominal muscle membrane of the frog. The muscles were tied over a glass tube which was filled with and immersed in Ringer solution. Electrodes of platinized silver on both sides of the muscle membrane connected the solution with the bridge. The stimulation was indirect.

The experiments showed that stimulation increases the resistance and the capacity of polarization. However, the interpretation of this result as a decrease of permeability is not the only one. It would be possible for the contraction of the muscle to diminish the interspaces between the muscle fibres. But this objection is answered by the following considerations: (*a*) that isometric contraction had the same effect, and (*b*) that the contraction outlasted the change of resistance and of capacity of polarization. The result suggests the explanation that the condensing of the membranes is effected by the formation of metabolic products. Further, the possibility that stimulation and contraction may be accompanied by opposite changes of permeability must be taken into account; it may be that the short process of excitation increases, while the longer-lasting process of contraction decreases, the permeability.

In a second communication the hypothesis just mentioned, relating to the influence of metabolic products, is examined by Quensel. The experiments show that during from one and one-half to two hours after the preparation of the muscles the resistance and the capacity of polarization fell, probably as an effect of injury to the plasma membranes. During this fall, $M/1000$ potassium cyanide had no influence. However, if the whole frog was poisoned by a sufficient amount of cyanide before the muscle membrane was prepared, the decrease of capacity of polarization was strongly diminished. It follows that cyanide protects the muscle against injury.

Furthermore, creatine decreased the permeability of the muscle for a longer period—still more so did monobromoacetic acid—but the decrease did not run parallel to the contracture. On the other

hand, sodium lactate increased the permeability (pH unchanged). Therefore, as is to be expected, lactate was able to compensate totally the densifying effect of creatine and monobromoacetic acid. Phosphate buffer, also, decreased the permeability a little; adenylic acid, applied as "lacarnol," gave a strong effect.

Kidney.—With the same method Plügge has examined the isolated kidney of the frog during arterial perfusion with Ringer solution. The electrodes (1.5×10 mm.) were put on the anterior and posterior surfaces of the gland. A striking parallelism between the rate of secretion and the electric behavior resulted; the increase of secretion was accompanied by the rise of capacity of polarization and vice versa. Thus, perfusion with $M/1000$ cyanide was followed by oliguria during from five to ten minutes, at the same time the resistance decreased. Thereafter the reverse happened; the oliguria was replaced by diuresis and the capacity of polarization rose. Pituglandol had nearly the same effect. After caffeine, resistance and capacity of polarization rose quickly, but the change was only partially reversible. The actions of ethylcarbylamine and of monobromoacetic acid were rather insignificant.

The author propounds the question as to whether the electric behavior is conditioned by opening or closing of short circuits due to different amounts of secretion inside the tubules or by different widths of blood vessels. The first objection, however, is discounted by observations on the effect of experimental slowing of the secretion; the second, because a parallelism between the circulation rate and the changes of polarization is lacking. According to the opinion of the author the often observed parallelism between increase of permeability, as measured by the capacity of polarization, and decrease of secretion can be considered as an argument for the theory of reabsorption in urine formation.

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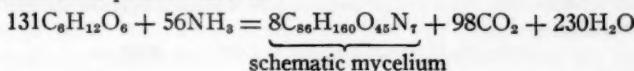
BIOLOGICAL OXIDATIONS AND REDUCTIONS*

BY RENÉ WURMSER

*Directeur du Laboratoire de Biophysique
de l'École des Hautes Études, Paris*

THE ENERGETICS OF OXIDATIONS AND REDUCTIONS

Some years ago (1), the reviewer showed why it is necessary to distinguish between oxidations associated with syntheses (oxidation-reductions) and those independent of syntheses. The fixation of free oxygen or respiration must be included among the latter type whenever it is not associated with an oxidation-reduction (Meyerhof's reaction). On the other hand, it was pointed out that alcoholic fermentation as well as respiration, properly speaking, appear to be reactions energetically independent of syntheses, or, to speak physiologically, independent of growth. It is thus necessary to single out of all the reactions which occur in an organism those which correspond to an increase in living matter. We have little experimental data on this subject. The case which has been most studied is that of the development of a mold, *Aspergillus niger*, which has already been the object of research by Molliard (2) and by Terroine and Wurmser (3). The last-mentioned authors were led by their thermochemical balance sheets to conclude that the synthesis of the mycelium resulted from the reduction of the carbon source (glucose) by a certain quantity of the same substance undergoing a partial and coupled oxidation. The question arises as to whether, in the course of this partial oxidation, there is an output of carbon dioxide. It does appear that in the course of growth the CO₂ production is in excess of that which would correspond to the maintenance of the mycelium already formed. But this excess may be due to secondary reactions—to the combustion of unutilized intermediate products. In agreement with this conception is the fact observed by Windisch (4), that yeasts in process of growth consume more oxygen than cells already formed. Tamiya (5) believes that the coupled reaction releases carbon dioxide. This author supposes, for example, that growth on glucose takes place as follows:



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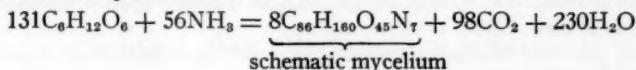
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This reaction corresponds to an increase of the respiratory quotient during growth. The equation permits us to calculate the volume of CO_2 corresponding to the formation of one gram of mycelium. Let λ represent this volume. Then the total respiratory quotient is given by the following relationship:

$$\text{R.Q.} = \frac{\Delta_m\lambda + Q_{O_2}(CQ)}{Q_{O_2}}$$

where Δ_m is the quantity of mycelium formed in a given time, Q_{O_2} the quantity of oxygen fixed during the same period, and CQ the ratio $\text{CO}_2 : \text{O}_2$ corresponding to complete combustion of the substrate (in the case of glucose, $CQ = 1$).

For other sources of carbon, for example ethyl alcohol, a lower quotient ($\text{R.Q.} < CQ$) is obtained instead of a higher quotient.

The values thus calculated are in good agreement with the values found experimentally in growth on various substrates¹. It is evident, however, that these formulae, which are reminiscent of the Armand Gautier¹ formulae, can in reality be established only by means of a minute analysis of all the residues of the culture and a very precise determination of the quantity of CO_2 corresponding to the maintenance of the mycelium.

Interest in the determination of thermochemical balance sheets carried out hitherto resides in what they indirectly teach us relative to the nature of the stoichiometric reactions which participate in growth.

However, the knowledge of these gross reactions is only a first step. In the present state of biochemistry this mode of representation is rather out of date. We must try to obtain a more precise knowledge of the intermediate mechanisms, to learn at what stages in the

¹ Tamiya believes that when physiologists speak of the energy necessary for a synthesis, for example, for that of fats from sugars, they fail to realize that this synthesis, when not isolated, always resembles a spontaneous reaction. One has only to read the famous discussion between Berthelot (6) and Pasteur to be convinced that Tamiya's belief is unjustified. The problem, which was stated particularly well by Gautier (7), is to identify the reactions associated with this reduction, which, with an equal number of carbon atoms, could not take place spontaneously. One should also compare Tamiya's equation for the synthesis, glucose → fat:



with the classic equation:



evolution of the glucides the coupled reactions begin from which the synthesis of living matter results, as has been done successfully in the case of the synthesis by fermentation of alcohol, glycerin, and lactic acid. It seems likely, for example, that the formation of alanine occurs through amination of pyruvic acid. Glucose or a derivative of glucose is oxidized to compensate for this reduction. The synthesis of living matter is indeed the sum of all these reactions, but what should be examined more closely is the specific natures of these partial reactions to discover the thermodynamic and kinetic conditions for their realization.

With regard to their kinetics it is known that the coupling of oxidations and reductions takes place, in all probability, by means of dehydrases, but the manner of intervention of a certain number of "growth factors" is unknown, and may be in some manner related to these catalysts. Thermodynamically, the problem is to find out whether the free-energy efficiency, that is, the ratio of the free energy corresponding to reduction to that of oxidation, is close enough to unity; or, more simply, whether the variation in free energy corresponding to an oxidation-reduction is small enough to permit the reaction to be considered as an equilibrium reaction. This is the essential question.

We are still very far from being able to answer it in relation to the numerous reactions, still very imperfectly known, which characterize the growth of an organism such as *Aspergillus*. However, some success has been attained in the discovery of numerous equilibrium reactions which are biologically important.

It appears from the calculations made by Burk (8) that this is the case, for example, in the assimilation of CO₂ by the autotrophic organism *Bacillus pycnoticus*, whose metabolism has been exhaustively investigated by Ruhland (9).

Other very important equilibria, which, however, are not oxidation-reductions, have been discovered by Meyerhof and Lohmann (10). The endothermic and non-spontaneous synthesis of phosphagen (creatine phosphoric acid) is made possible in muscle by coupling with the exothermic and spontaneous decomposition of adenylylpyrophosphate; resynthesis of adenylylpyrophosphate from adenylic acid and inorganic phosphate is in turn effected by the energy of formation of lactic acid. If it is admitted that this coupling takes place through the transformation of hexophosphate, we see that the phosphoric-acid group is common to all these reactions. We can thus understand how it is that adenylylpyrophosphate acts as a coenzyme in

the formation of lactic acid, since the reception of phosphoric acid by hexose would then be related to the liberation of phosphoric acid from the adenylylpyrophosphate or the yielding of phosphoric acid by the ester with reconstitution of adenylylpyrophosphate.

But these equilibria, as has already been stated, do not pertain to oxidation-reductions—they are esterification reactions. It is this type of transformation which concerns the anaërobic phase of contraction. With respect to the processes of aërobic recovery in which reconstitution of hexose from lactic acid intervenes (Meyerhof's reaction), this reaction occurs under conditions remote from reversibility.

We shall not discuss at greater length the equilibria of muscular contraction, since these are not directly concerned with the chemistry of oxidations. On the other hand, certain reversible reactions are of special interest from this point of view; these are reactions involving so-called *electroactive* bodies of metabolism, that is, bodies which are able to exchange an electric charge with an inert electrode. In a recent paper (11) it has been shown that account should be taken of various oxidation-reduction levels in the cell, as determined by the characteristic potentials of the electroactive systems in the cell. The system of greatest mass tends to impose its potential on the whole and governs the direction of the reactions corresponding to the other coexisting systems. It is therefore important to determine the characteristic potentials of the various constituents of the cell.

The progress of these investigations is closely allied to studies on oxidation-reduction dyes. Cognizance should therefore be taken of certain researches in which Mansfield Clark has been the pioneer. Thus, Clark and Perkins (12) have made an extensive study of the potentials of neutral red, and have shown that neutral red, although behaving as a reversible system, shows peculiar secondary changes in the pH range of physiological interest. Michaelis (13) has described a new dye, viologen or dimethyl- $\gamma\gamma'$ -dipyridylchloride, which is of great interest because its normal potential is very negative ($E'_o = -0.44$ volt at pH 7, i.e., 0.02 volt more negative than that of the hydrogen electrode at the same pH).

Some considerations of great interest to the determination of cellular potentials are to be found in Barron's work (14) on the rate of autoxidation of oxidation-reduction systems in relation to their free energy. At constant pH and in the absence of catalysts it has been found that there exists a linear relation between the normal potential E'_o of the dye and the logarithm of the time necessary to oxidize the dye from 2 per cent to 50 per cent oxidation. As E'_o

becomes more positive, the time necessary to oxidize the reduced dye increases. Although this rule may not be entirely without exception, it enables one to interpret the measurements made with the dyes in the presence of air over periods of some length. The dyes are then subjected to oxidation by air and reduction by the constituents of the cytoplasm, and for each dye a specific regimen is established. Since the rate of oxidation increases and the rate of reduction diminishes as the potential of the dye becomes more and more negative, the position of a partially reduced dye in the scale of indicators can inform us quantitatively, if not simply, concerning the reducing systems of the cell.

With respect to the oxidation-reduction levels of the cellular constituents themselves, considerable difficulty exists. Among the electroactive bodies we may mention hexuronic acid, which to all appearances seems to be vitamin C. Some authors had already found correlations between the distribution of the vitamin and the reducing factor of cells. Svirbely and Szent-Györgyi (15) now seem to have established the fact that hexuronic acid, discovered and isolated from plants and the adrenal cortex by Szent-Györgyi at the Biochemical Laboratory of Cambridge in 1927-1928, is vitamin C. This hexuronic acid is susceptible to reversible oxidation, and Georgescu (16) has determined its oxidation-reduction potential. He has established the fact that hexuronic acid extracted from orange juice presents a well-defined oxidation-reduction potential. The characteristic potential is situated in the region corresponding to 0.030 volt at pH 7. He has, furthermore, investigated the oxidation-reduction potential of glycuronic and galacturonic acids and has found that these acids also have a characteristic potential situated in the same region.

Ball and Clark (17) have undertaken the study of the oxidation potential of epinephrin (adrenalin) and catechol by the use of ceric sulphate and potassium ferricyanide. Orthoquinone is obtained as the product of oxidation of adrenalin, but the orthoquinone ultimately suffers decomposition, caused by the ionizable side chain, in a manner similar to that which has been shown for the oxidation product of homogentisic acid. Nevertheless, the characteristic potentials may be arrived at by extrapolation.

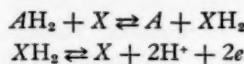
Ball and Chen (18) have measured by the same method the oxidation-reduction potentials of several systems, the oxidants of which decompose rapidly — dihydroxyphenylalanine (dopa), adrenalone, protocatechuic acid, ethyl ester of protocatechuic acid, and gentisic acid. A number of these substances occur naturally. It may readily

be seen that if they were not held in a reducing environment they would soon suffer destruction. Such a reducing tendency must exist in the tissues where such compounds are found. This is in harmony with direct and other indirect measurements of the potentials of living cells.

But the study of the potentials of cellular constituents should not be limited to bodies electroactive in themselves; for substances which are not in themselves electroactive may enter into electrochemical equilibrium within the cell in the presence of enzymes and of electroactive substances.

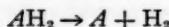
Quastel and Whetham (19) on the one hand and Thunberg (20) on the other, as well as later investigators, have determined the oxidation-reduction potential of a mixture of succinic acid and fumaric acid in the presence of biological catalysts and methylene blue. Up to the present, among all the oxidation-reductions which have been realized in the presence of enzymes, this equilibrium between succinic and fumaric acids was the only one which had been quantitatively established. However, the theory of catalysis indicates that in each case the regressive reaction ought, in the vicinity of the equilibrium point, to occur with the same speed as the progressive reaction. The method which has permitted the demonstration of the existence of the equilibrium in the case of succinic acid is a general one, but the limits within which it may be utilized are quite narrow.

To determine the oxidation-reduction potential of a system consisting of the substance AH_2 , which is inactive towards an inert electrode, and its oxidation product A , it suffices in principle to make it react by means of a suitable catalyst with another electroactive body X —that is, a body which is capable of exchanging electrons with an inert metal, and which is itself in equilibrium with its reduction product XH_2 . We then have the series of reactions



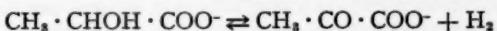
e representing an electron.

The determination of the potential therefore furnishes the value of the ratio $[XH_2] / [X]$ and thus permits the calculation of the ratio $[A] / [AH_2]$, from which we get the change in free energy for the reaction



The body X must, however, satisfy one condition. It is essential that the normal potential E_0 of the system X, XH_2 be very close to

that of the system A, AH_2 . Working with cresyl violet as the electro-active intermediary, Wurmser and Mayer (21) have been able to attain in both directions the equilibrium point for the reaction



They found that the normal potential is very nearly -0.200 volt at $37^\circ C.$ and pH 7.2 (that is, $+0.245$ volt at pH 0).

Baumberger, Jürgensen, and Bardwell (22) have also sought to determine the oxidation-reduction potential corresponding to this same equilibrium. They find that between pH 5.2 and 7.2 the potential has a constant value. The normal potential at pH 0 is $+0.316$ volt at $32^\circ C.$ This value is more positive than the preceding one, no doubt because the indicator utilized is too positive relative to the system lactate \rightleftharpoons pyruvate.

At pH 7 it is found that for a hydrogen activity corresponding to rH_6 pyruvic acid fixes hydrogen and is converted to lactic acid. It is furthermore known that the cells of aerobic organisms acquire a potential corresponding to rH_6 when they are maintained in anaërobiosis [Aubel and Lévy (23)]. This value may be explained by the average concentration of the glutathione in the cells. It may be objected, however, that it ought also to appear in cells containing hydrogen donators, such as the aldehydes, which are capable of reducing all oxidation - reduction indicators, including neutral red. One is therefore tempted to see in the formation of lactic acid at the expense of pyruvic acid one of the reactions which determine this limiting potential.

In this connection the work of Barrenscheen and Beneschovsky (24) is of great interest. It is generally admitted, owing to the works of Neuberg and Kobel (25) [see also Simon (26)], that the transformation of methylglyoxal to lactic acid takes place by means of a Cannizaro reaction. However, another possibility is that the methylglyoxal is oxidized to pyruvic acid² and then hydrogenated with formation of lactic acid. Barrenscheen and Beneschovsky have now shown that this reduction may in fact take place, thanks to the sulphydryl compounds of the cell.

Although we do not know with certainty the oxidation-reduction level corresponding to the synthesis of amino acids from their oxidation products, it is known that it is sufficiently positive for the cell

² A direct oxidation of methylglyoxal-bisulphite to pyruvic acid by oxygen has been demonstrated by Neuberg and Kobel (27).

to have a constant tendency to reduce it, that is, to resynthesize the amino acid. Some recent investigations by Schuwirth (28), resulting from those of Wieland and Bergel (29) and of Kisch (30), show that various amino acids, such as glycine and alanine, are deaminized in the presence of palladium black and various acceptors in the absence of O_2 . Other acceptors, such as methylene blue, alloxan, and isatin, are not reduced. It may be supposed that acceptors which are not reduced are precisely those whose oxidation-reduction potentials are too negative, which would be in good agreement with our present knowledge of this subject.

THE ORIENTATION OF REACTIONS³

An important work by Windisch (4) relative to this topic reopens an old controversy. It is known that Pasteur has shown that yeast produces less alcohol at the expense of the same quantity of sugar when in contact with air than when there is an absence of oxygen. However, although Pasteur saw the problem perfectly [see Schoen (31)], he was not able to establish the fact that the rate of fermentation, as measured by the quantity of alcohol formed per unit weight of yeast, is lowered in the presence of air. The demonstration of this fact is due to Meyerhof (32). However, Windisch, after confirming the fact that all species of yeast undergo an increase in fermentative capacity in the absence of air, observed another fact, namely, that the action of oxygen is effective only during the process of multiplication. When the yeast is no longer multiplying, the fermentative capacity is not influenced by the presence of oxygen, and under certain conditions is even distinctly increased.⁴

In this connection it would be of interest to calculate the ratio of the weights of non-fermented sugar to the weight of yeast formed and to compare it to the ratio obtained in the growth of *Aspergillus niger*; for oxidation-reductions of the same type must occur in both cases to effect the synthesis of living matter. A certain quantity of glucose undergoes alcoholic fermentation; another portion serves as raw material for the syntheses; a third serves as a reducing agent in the latter process; a fourth is oxidized according to the respiratory

³ Cf. Wurmser, R., *Ann. Rev. Biochem.*, 1, 61 *et seq.* (1932).

⁴ During the correction of these proofs there appeared a paper by Kluyver & Hoogerheide [*Compt. rend.*, 196, 443 (1933)]. These authors show that in Windisch's experiments the conditions were such that oxygen was not in sufficient quantity to exert an unfavorable action on the fermentative process.

type; and, finally, a fifth fraction is oxidized, no doubt, as in the case of *Aspergillus*, in the course of the secondary reactions indirectly connected with the synthetic processes.

The competition between molecules about to undergo fermentation, total oxidation, and utilization in syntheses depends here especially on catalytic factors. It is very interesting to observe that we must count among these factors the very oxygen which favors the multiplication of cells and, consequently, the oxidation-reductions bound up with the syntheses. Here the oxygen, according to an old conception of Pasteur (33), acts in a manner different from that of oxygen entering into the reaction, that is, it acts catalytically.

As for the yeast already formed, the orientation which it gives to the glucose in the direction either of fermentation or of ordinary respiratory oxidation depends on the proportion of catalysts corresponding to these two types of transformation. Schoen (34) rightly observes that, generally speaking, it is not alone the presence of a catalytic system within the cell which characterizes its biochemical specificity. It is, above all, essential that its functioning be not interfered with by another catalytic system. For example, yeast breaks up the methylglyoxal molecule. In order to produce alcohol and carbon dioxide, the catalyst which permits this operation must predominate over the ketoaldehyde mutase which would transform the methylglyoxal into lactic acid. Likewise, lactic specificity is due to a deficiency of catalyst capable of catabolizing this intermediary substance; similarly, fermentation would be due to a deficiency of oxidation catalysts.

The work of Windisch shows, furthermore, the influence of the culture medium on the formation of these various catalysts. He shows that an anaerobic yeast culture enhances equally the respiratory and fermentative capacities of the cells. There is a general heightening of all the vital functions of the yeast. It is as though in the process of anaerobic culture there were formed, perhaps owing to the oxidation-reduction potential which anaerobiosis confers on the culture medium, more abundant catalytic systems.

Another question then arises which is closely allied to the study of the intermediate metabolism, and for that reason we shall give a brief indication of it. Given a cell which, thanks to its catalytic systems, can oxidize glucides, it must still be asked where the deviation between the fermentative processes and the respiratory processes begins. In last year's *Review* we cited numerous investigations of this problem. One of the first ways of attacking the question is to

compare the CO_2 production in the absence of air (or after poisoning of the catalysts) with the CO_2 production in the presence of air under normal conditions.

Pei-sung Tang (35) has shown that the consumption of oxygen by germinating seeds of *Lupinus albus* can be reversibly inhibited by CO to a maximum extent of 36 per cent, using a mixture of 24 per cent O_2 and 76 per cent CO at 18°C , in darkness. It is found that the production of CO_2 is apparently not inhibited by CO and that the rate of CO_2 production in N_2 is slightly lower than that in air. The author concludes that it is unlikely that the anaërobic production of CO_2 is replaced quantitatively by an aërobic CO_2 produced through an essentially different mechanism.

This conclusion thus favors the identity of the first stages of the attack on the molecule, whether it be destined towards fermentation or towards combustion by molecular oxygen. As is well known, this point of view has encountered objections by Lundsgaard.

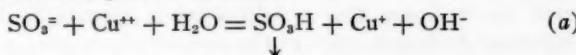
Iodo- and bromoacetic acids can stop, at relatively low concentrations, the anaërobic scission of glucose and equally well the formation of lactic acid. This is true of animal tissues and of alcoholic fermentation by living yeasts or zymase preparations. Respiration remains practically unchanged at a suitable concentration of the inhibitor. Yamasaki (36) has established the fact that hexosediphosphate in the presence of sodium moniodoacetate is capable of fermenting; in this case it is the phosphorylation of glucose which is inhibited by the poison. The same result appears in the work of Lohmann (37). Lundsgaard (38) in a new investigation confirms his earlier conclusions, namely, that yeast in which anaërobic degradation of the glucides has been arrested can catabolize these glucides aërobically, but he recognizes that their pure oxidation appears to be less extensive than was formerly supposed.

REACTION VELOCITY FACTORS—CATALYSTS

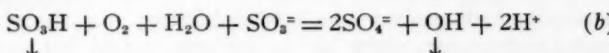
It remains for us to review the investigations relating to the nature of those catalysts which, as we have seen, often play an essential rôle in the orientation of cellular reactions.

With regard to the action of enzymes in general, a very interesting theory has been forwarded by Haber and Willstätter (39). This theory has its point of departure in the work of Frank and Haber on autoxidation of alkaline sulphites. These authors have shown that

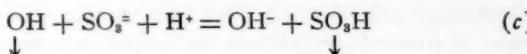
when this reaction, which is a chain reaction, is initiated by copper salts there first takes place the reaction



The radical constituted by the monothionic acid then reacts according to Equation *b*:

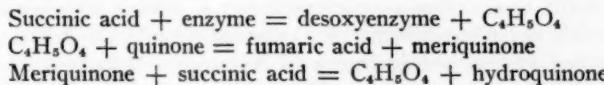


The new hydroxyl radical thus liberated then reacts according to Equation *c*:



According to Haber and Willstätter the action of an enzyme may likewise be represented as giving rise to monovalent radicals which react afterwards.

For example, in the oxidation of succinic acid the first radical to be formed is monodehydrosuccinic acid ($\text{C}_4\text{H}_5\text{O}_4$) ; the second radical is a meriquinone:



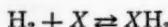
The reaction continues in this manner without further intervention of the enzyme as long as the chain is not interrupted, as, for example, by the union of two radicals.

Haldane (40) has shown certain difficulties presented by this theory. He says: "If the chains end by the collision of free radicals, we should expect the reaction velocity to vary as the square root of the enzyme concentration instead of being proportional to it. If they include free radicals, such as OH and meriquinoids, the specificity of enzymes as regards the activation of reducing substrates is hard to explain."

Whatever be the mechanism of catalysis by dehydrases, the importance of their rôle in the general chemistry of the cell becomes more and more apparent. We know that according to Wieland's theory, a typical example of which has been brought forward by Bertho (41) in his work on lactic-acid bacilli, and which finds further confirmation in the work of Müller (42) on acetic bacilli, the

basic fact of oxidation is a mobilization of hydrogen. Thunberg's dehydrases are the enzymes which catalyze this mobilization. However, the hydrogen thus mobilized rarely appears in the guise of molecular hydrogen, even in the absence of oxygen; and it is not always because the hydrogen thus liberated is in equilibrium with the substrate at a very low tension.

The experiments of Stephenson and Stickland (43) show that molecular hydrogen must be produced, owing to a special enzyme called hydrogenase, which has not yet been isolated, but which catalyzes the reaction



Stephenson and Stickland (44) have likewise described, under the name of formic hydrogenlyase, the enzyme, also not extracted, which catalyzes the reaction



It appears by adaptation when *coli* are cultured in the presence of formate.

In general, the mobilized hydrogen fixes itself on transporters which, in turn, reduce either the molecular oxygen or other molecules. It is in this manner that a considerable part of respiration takes place, according to Keilin's schema (45); thanks to the Wieland-Thunberg dehydrase system, the hydrogen of metabolites reduces molecular oxygen in the presence of an oxidase (the so-called *Atmungsferment* of Warburg).

The same schema provides for transfer by hexuronic acid and the phenols [Szent-Györgyi (46)].

Finally, glutathione can now be considered, thanks to the researches of Mann (47) and Meldrum (48) as apt to function in the same way.

Since its isolation by Hopkins (49), it has been assumed that glutathione acts as a catalyst of tissue respiration. But the only tissue system known before now which reduced glutathione was the thermostable residue of Hopkins and Dixon. With thermolabile systems (dehydrase action) only negative results were obtained. Mann demonstrates the very important fact that glutathione is reduced slowly by glucose in the presence of glucose dehydrase prepared from liver by a method similar to that of Harrison (50), and he also shows that this reaction is greatly accelerated by an activator, soluble in water, which can be extracted from liver.

The reduction of glutathione is thus included in the general schema; as to its oxidation, it is known that this is activated by the heavy metals, in accordance with Warburg's conception.

In the same manner one is tempted to conceive of the new ferment which oxidizes hexosemonophosphate in the presence of methylene blue or air, and which has just been discovered by Warburg and Christian (51) in bottom yeasts, as perhaps made up of one part which is truly enzymatic and a transporter. This new ferment, the absorption spectrum of which Warburg & Christian have determined, is reduced by the cell donators. This reduced form (colorless) can be oxidized in the presence of an acceptor such as methylene blue as well as directly by oxygen, without the intervention of a haemin catalyst.

The general schema which implies the mobilization of hydrogen by dehydrases, its transfer, and the oxidation of the transporter by a hydrogen acceptor (which in the case of respiration is molecular oxygen generally mobilized by an oxidase system) may vary in complexity from case to case. It seems that if the hydrogen serves to reduce molecules other than oxygen the transporter may not be essential, and an enzyme may catalyze the transfer of hydrogen directly.

As concerns the dehydrase system, we have just seen that, in the case of glutathione, reduction could be effected with glucose in the presence of dehydrase only if a coenzyme was added. In yeast, the existence of a co-reductase [Euler and Nilsson (52)] is more difficult to prove. On the other hand, in the respiration of muscle the coenzymes which intervene are in the process of being identified.

Lohmann (53) has succeeded in showing that the complete coenzyme system for lactic-acid production in tissues is the complex $Mg + \text{adenylpyrophosphoric acid}$ (adenosinetriphosphoric acid). According to Lohmann, this complex is not the same as cozymase [but see Euler and Nilsson (54)].

The similarity in the anaerobic metabolism of glucides in muscle and yeast leads to the belief that the coenzyme acts in both cases on the same phases of transformation. Moreover, from the unitary theory of metabolism, according to which respiration and fermentation have common initial stages, it might have been expected that the coenzyme of lactic-acid fermentation would be identical with that of respiration at the expense of glucides. But from the work of Banga and Szent-Györgyi (55) it seems to follow that the coenzyme, that is to say, the adenylpyrophosphoric acid, plays a definite rôle only in

that part of the respiratory oxidations which is non-sensitive to poisoning by arsenious acid, that is, the smallest part of the respiration. Further, this coenzyme accelerates the oxidations of washed muscle only at the expense of zymophosphate (lactacidogen and hexosediphosphate). The lactic acid and the glucose are inactive. Therefore the coenzyme seems to act, in this case at least, only on the cleavage of hexosephosphates.

Another coenzyme functions in that part of respiration which is inhibited by arsenious acid. This coenzyme permits the muscle to act not only on lactacidogen but also on lactic acid. In the latter case phosphorylation does not intervene.

We have thus seen a dehydrase system which presents great complexity. As for the oxidase system, it seems to be simpler. However, there still remains the task of elucidating the physiological rôle of peroxidases.

Elliott (57) makes the attractive hypothesis that peroxidase may be simply a counterpart to Keilin's indophenoloxidase, using H_2O_2 instead of O_2 as the oxidizing substrate, and requiring the activation by other enzymes of most of its organic reducing substrates, and possibly the mediation of cytochrome as well. Keilin has found that cytochrome is oxidized by H_2O_2 alone, but this oxidation may be accelerated by peroxidase. Elliott has studied the activity of milk peroxidase on various substances—formate, acetate, oleate, stearate, triolein, hydroxybutyrate, glucose, glycerol, acetaldehyde, lactate, ethyl alcohol, glycine, phenylalanine, histidine, dihydroxyacetone, phenylglyoxal, tryptophane, and tyrosine. Only the last two substances were found to be oxidized by H_2O_2 with peroxidase catalysis.

In the presence of horse-radish peroxidase, Elliott (58) found that nitrite and tryptophane are not oxidizable by H_2O_2 . This contrasts with the behaviour of milk peroxidase. In Elliott's opinion these results throw no light on the rôle of peroxidase as a constituent of the respiratory mechanism.

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THE CHEMISTRY OF THE CARBOHYDRATES AND THE GLYCOSIDES*

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INTRODUCTION

The activity in the field of sugar chemistry continues to be greater than in any other single field of organic or biochemistry, making the task of the reviewer a very difficult one. It is gratifying, however, to note that the principal objectives of the work of recent years now appear within reach, and that, although controversies are not entirely eliminated, they are no longer very acute.

The problem of the ring structure of the normal and γ -glucosides seems to be settled in favor of the views reached by the methods of organic chemistry. Further, a physical method was introduced by Rüber for determining the ring structure of non-substituted sugars.

The problem of the structure of the known di- and trisaccharides has been practically solved, and the past year brought evidence in confirmation of the earlier conclusions reached through the method of synthesis.

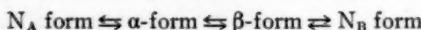
The greatest divergence of views existed on the question of the structure of the polysaccharides of higher molecular weight such as cellulose, starch, inulin, etc. Only a few years ago it seemed as if the older views which regarded these substances as built of their structural units, the monosaccharides, through their primary valences was to be abandoned in favor of a structure maintained through molecular forces. In course of the last year the greater number of publications upheld the classical theory, although in a minor degree dissension still persisted.

However, even the complete solution of all the structural problems would not mean an end to sugar chemistry, for already there are indications that sugar chemists are turning their attention in a new direction, toward the application of purely chemical methods to problems closer to those of the biochemists, namely, toward the mechanism of biological sugar degradation.

* Received February 16, 1933.

I. GENERAL

Isomers in solution.—The question of the number and character of the different isomers existing in an aqueous solution of a given sugar was discussed by Lippich (9). The method of investigation consisted in measuring the HCN binding capacity of different sugars as a function of the hydrion concentration. In the case of the α -isomers, the binding capacity increases with the progress of mutarotation; in the case of the β -forms, the reverse takes place. The author is inclined to believe that there exist in an aqueous solution four isomers:



The N forms are regarded as active aldehydic forms, having stereoisomeric properties so that N_A passes into the α - and N_B into the β -pyranose form. No details as to the nature of the stereoisomerism of the N_A and N_B forms are given. He assumes that the active forms may isomerize into one another without passing through the cyclic forms and that the latter may isomerize into one another without passing into the aldehydic form.

From a study of the variations of rotatory power and solution volume with time, Riiber (13) concludes that an aqueous solution of arabinose contains three modifications. A new form was prepared with a rotation higher than that previously recorded for arabinose.

Isorotation.—A new criticism of Hudson's isorotation principle was advanced by Freudenberg & Kuhn (4). The object of the article was to prove the untenability of the isorotation principle on the grounds of the physical theories of optical rotation. Hudson's views are based on an acceptance of the unrestricted validity of the superposition principle. Fundamentally, Freudenberg & Kuhn have no quarrel with this principle as a first approximation, and in an earlier article Kuhn, Freudenberg & Wolf accepted and explained it in terms of the physical theory. However, an analysis of the optical rotations of certain monocarboxylic sugar acids showed definite lack of agreement between the observed results and those expected according to the superposition theory. The invalidity of the superposition theory in the case of the sugar acids was explained on the assumption that two groups, differing from each other in spatial sense alone, may have an entirely different vicinal effect on the other groups attached to the same carbon atom. This influence may become negligible only in those cases in which the second asymmetric center is far removed

from the principal asymmetric carbon atom. In their concluding paragraph the authors state: "Among all the observed substances no one case was found in which stereoisomeric differences of the carbon atoms attached directly to the asymmetric carbon atom furnishing the principal contribution did not display a different vicinal effect on that group." And further: "The cases in which the superposition principle was found to hold are characterized by the fact that the stereoisomeric differences were observed on carbon atoms at least once removed from the first asymmetric carbon atom."

Another criticism of the isorotation rule is contained in the article by Harris, Hirst & Wood (6). These authors made a study of the rotary dispersion of the furanosides and pyranosides of mannose and of glucose. The wave-lengths employed extended from λ 7520 to λ 2614. In general, the deviation from simple dispersion was small in the visible spectrum, yet many of the substances fail to conform to Hudson's isorotation rules. In general, the authors concluded that, in the case of substances possessing the configuration of mannose on the first three carbons, Hudson's rules cannot be applied.

Georg (5) prepared and studied the α - and β -isomers of tetracetylglucose. The first was prepared essentially by the method of Nef, who had heated an ethereal solution of acetobromoglucose with silver nitrate. The tetracetate obtained by Nef should be regarded as the α -isomer, for its properties are very similar to those of α -tetracetylglucose subsequently prepared by Schlubach. When the reaction is performed in rigorously dried ether, β -acetonitroglucose is formed. In the presence of moisture, however, Georg found that α -tetracetylglucose predominated, accompanied by the β -tetracetate and by α -acetonitroglucose. The β -tetracetate was prepared by Georg by the method of Fischer & Hess, but was brought to a higher degree of purity than by previous workers. The two isomers were then used to compare the rotatory contribution of carbon atoms 2, 3, 4, 5 of this new pair of acetates with the values obtained by Hudson & Dale in the case of the tetracylmethylglucosides and the pentacetylglucoses. The conclusion was that in the case of the tetracetates the value of 2 B in chloroform was 52,200 as compared with 40,700 for the tetracylmethylglucosides and 41,000 for the pentacetates. On the other hand, the value for acetochloroglucose is 54,000. Hence, the author concludes that the vicinal effect of the OH group of carbon atom 1 is similar to that of the Cl atom in the same position.

The ethyl glucosides and their acetates have been prepared by

Ferguson (3), and their rotations have been measured. It is concluded that the results confirm Hudson's first and second isorotation rules.

Mutarotation.—The influence of acid on the mutarotation of glucose has been examined by Schenk (14) for two wave-lengths and with different concentrations of acid. A constant difference in rotation for the two wave-lengths was found for the lower acid concentrations, becoming larger for the higher concentrations. Mutarotation was not observed in solutions containing more than 3 per cent acid, either because it was too rapid to be observed or because it was absent.

Zerner's theory that mutarotation of osazones is due to tautomerization between hydrazine and azo form is shown by Votoček & Valentin (16) to be untenable, for fructose methylphenylosazone, possessing no mobile hydrogen, still shows mutarotation.

The mutarotations of phenylhydrazone of mannose and rhamnose were studied by Butler & Cretcher (1). The authors found that these substances, contrary to statements in the literature, do mutarotate but in a very unusual manner. This finding was not surprising inasmuch as the hydrazone are capable of undergoing isomerizations of various types. In a second paper (2) on the *o*-, *m*-, and *p*-nitrophenylhydrazone of mannose and rhamnose, the same authors have tested the rule formulated by Frankland & Cohen for the effect of position isomerism on optical rotation, inasmuch as mutarotation has not been taken into consideration in past work. The present authors conclude that it is futile to attempt to apply rules of position isomerism to a system as complex as that of solutions of sugar hydrazone in which many isomerizations are possible.

Miscellaneous.—Two papers appeared during the year dealing with methods for determining primary hydroxyl groups. One by Hockett & Hudson (7) reports that α - and β -methylxyloside, α -methyllyxoside, and β -methylarabinoside react with triphenylmethyl chloride as shown by polarimetric change. The reactions took from 100 to 360 hours, and yields of 45 to 80 per cent of amorphous product were obtained. This led to the conclusion that either this reagent is not specific for primary alcoholic groups, as believed by Helferich, or else that none of these glycosides has the $<1,5>$ ring. With α -methylmannoside a crystalline product was obtained and is being further studied.

The second paper is by Oldham & Rutherford (11). The authors

conclude that *p*-toluenesulfonylchloride may be used as a reagent for identification and estimation of the 6-hydroxy group in glucose. The *p*-tolylsulfo group, introduced in this position in the glucoside, reacts readily and quantitatively with sodium iodide in acetone, but if in other positions the rate is invariably far lower. The iodine introduced into position 6 during the reaction may be estimated quantitatively by boiling the substance with a solution of silver nitrate in acetonitrile solution. The nitrate is thus formed and may in turn be re-converted to the corresponding OH derivative. These reactions are peculiar to the 6-hydroxyl group.

The effect of a 2-*p*-toluenesulfonyl group on the reactivity of a glucosidyl halide was studied by Reynolds (12). The author found that introduction of this group into position 2 led to almost complete suppression of reactivity of the 1-chloro-group in 3,4,6-triacetyl- β -glucosidyl chloride, the substituted chloride being recovered unchanged on shaking the cold methyl-alcohol solution with silver carbonate or oxide. By boiling the methyl-alcohol solution with silver nitrate and pyridine, the glucoside is obtained, however. The tolylsulfo derivative in pyridine at room temperature was found to show no polarimetric change, whereas the original triacetylchloro compound, its 2-trichloroacetyl derivative, and acetobromoglucose, all showed large and relatively rapid changes of rotation in the same solvent.

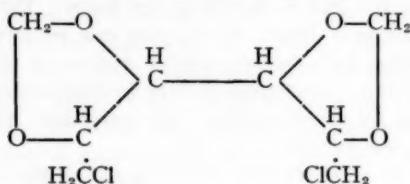
The acidic property of sugars has been studied by Urban & Shaffer (15) by determining the amount of sodium hydroxide neutralized by glucose, fructose, and sucrose. All three behave as dibasic acids, and dissociation constants are calculated. A third acidic group is stated to commence functioning at higher alkalinity, but its existence is regarded as uncertain. Stearn,¹ however, found at least five ionizing hydrogens in most sugars.

In order to determine the proportions of furanoside and pyranoside formed from the commoner monoses, Levene, Raymond & Dillon (8) followed the reaction of the sugars with methyl-alcoholic HCl, both optically and by an analytical method which was developed. No direct relation between configuration and rate of glucoside formation was secured. Considerable optical and analytical data are presented.

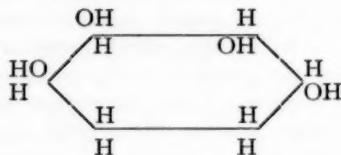
A successful transformation of a hexose derivative into a cyclitol

¹ *Ann. Rev. Biochem.*, 1, 214 (1932).

was accomplished by Micheel (10). The many attempts in the past to perform this biologically important reaction had met with failure. The starting material for the reaction was 1,6-dichloromannitol, whose dimethylene derivative has the following projection formula.



On treatment with very active silver, this substance formed a dimethylene cyclohexane, and on hydrolysis of the methylene groups the following cyclitol was obtained.

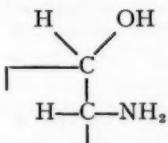


II. STRUCTURE

Configuration.—Auwers' observation that the refractivity of two adjacent carbon atoms having hydroxyl groups in *cis* position is greater than when they are in *trans* position induced Riiber (47) to compare the molecular refractivity of the α - and β -isomers of glucose. He found that the molecular refractivity of the α - form is 0.39 less than that of the β -isomer. Hence, he concluded that in the α -form the hydroxyls attached to carbon atoms 1 and 2 are in the *cis* position. This conclusion is in agreement with the generally accepted view. As refractivity is an additive property, the author was encouraged to think that the method may serve as a means of elucidating the configurations of sugars.

Some time ago Micheel & Micheel made the observation that trimethylamine reacts with halogenoacetyl sugars only in those cases where the halogen is in *cis* position to the hydroxyl of carbon atom 2. On substitution of the halogen, a Walden inversion takes place and the trimethylamine residue occupies a position *trans* to the hydroxyl of carbon atom 2. These observations have now been con-

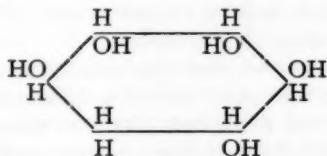
firmed (40). The authors then proceed to make use of these observations in elucidating the configuration of glucosamine in the following way: Acetobromoglucosamine hydrobromide, $[\alpha]_D = +128^\circ$ (CHCl_3), which is regarded by them as the α -isomer, reacts with trimethylamine to form a β -isomer, in which the trimethylamine residue is *trans* to the amino group of carbon atom 2. Hence, α -glucosamine has the configuration



The authors report this as a tentative conclusion requiring further confirmation.

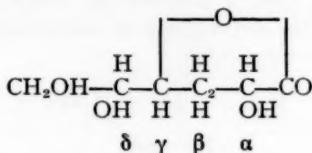
The effect of configuration upon reactivity is revealed by a study of acetyl derivatives of galactose. Schlubach & Prochownick showed some time ago that treatment of β -pentacetyl galactofuranose with liquid HBr replaces two OCOCH_3 groups by two bromine atoms with much greater velocity than in the case of pentacetyl glucopyranose. Thus the question arose as to whether the higher reactivity of the galactose pentacetate was due to the differences in the ring structure or to the differences in configuration. In a recent article Schlubach & Wagenitz (50) furnish evidence that the high reactivity is due to the stereochemical factor, inasmuch as pentacetyl galactopyranose reacts with the same velocity as the corresponding furanose derivative. Incidentally, the authors demonstrated that the new bromo-derivative is 1,6-dibromoacetogalactose, since the glycoside derived from it may be readily reduced catalytically to triacetyl-methylfucopyranoside.

For the configuration of *d*-quercitol, Posternak (46) gives the following formula:



The choice lay between four theoretically possible configurations, and the arguments in favor of the one chosen were as follows. On

oxidation by means of KMnO_4 (2.8 atoms of O) a trihydroxyadipic acid was obtained, identical with metasaccharonic acid (α -galacto-metasaccharonic acid). This in its turn is the oxidation product of the metasaccharin to which Nef assigned the following configuration:



Posternak confirmed this configuration by degrading metasaccharin (following Kiliani) to a desoxypentose which was found to be identical with the *d*-2-xylodesose of Levene & Mori. This established the configuration of the γ - and δ - carbon atoms and that of the α - was assigned on the basis of the phenylhydrazide rule. Thus, the metasaccharonic acid is formed by rupture of the quercitol ring between carbon atoms 3 and 4 or 4 and 5.

Ring structure.—A new method for the determination of the ring structure of simple sugars was developed by Criegee (23). It is based on the fact that α -glycols are readily oxidized by $\text{Pb}(\text{OAc})_4$ with the formation of aldehydes. Formaldehyde can be formed from CH_2OH groups when in the adjacent CHOH group the hydroxyl is not substituted. Hence, only aldohexofuranosides can yield formaldehyde on oxidation by this reagent. This expectation was realized on a series of sugars, the only furanosides from which formaldehyde is not formed being α -methyl-mannofuranoside and *l*- γ -mannolactone. The author explains these exceptions on the basis of the observation that in cyclic structures the neighboring *cis* hydroxyls are specially susceptible to oxidation with the above-cited reagent. The hydroxyls 2 and 3 in mannose present such a case and hence in this sugar these two hydroxyl groups oxidize preferentially. Indeed, addition of boric acid to form a complex with carbon atoms 2 and 3 increases the formation of formaldehyde.

Micheel had observed that glucosido-trimethylammonium bromide on treatment with alkali formed a 1,6-anhydride analogous to levoglucosan. Micheel & Micheel (39) have now attempted to prepare anhydrides with different ring structures, and sought to accomplish this by introducing the triphenylmethyl residue in position 6. They were particularly interested in preparing the α -<1,5>- β -

<1,3> anhydride, inasmuch as Tanaka had assigned this structure to the α -glucosan of Pictet. However, on treatment with aqueous alkali, no definite product could be obtained. On treatment with methyl-alcoholic alkali, 6-trityl- β -methylglucoside was obtained. If it is recalled that α -glucosan on opening the second ring leads to α -methylglucoside, then the possibility of the formation of the 6-trityl- β -methylglucoside through the intermediary of an α -<1,5>- β -<1,3> anhydride is excluded and the glucoside formation must be regarded as a primary reaction. Thus apparently when the possibility of formation of the <1,6> ring is excluded, no other ring is formed. Likewise, the attempt to obtain a second ring closure by the action of alkali on triacetyl- β -xylosido-trimethylammonium bromide or on triacetyl- β -isorhamnosido-trimethylammonium bromide was unsuccessful.

The β -glucosides of 3-methyl- and 4-methylglucose were prepared by Helferich & Lang (28), but neither was susceptible to hydrolysis by emulsin. The same authors prepared 2,4-diacetyl-3-methyl- β -methylglucoside-6-iodohydrin and 1,2-monoacetone-3,5-diacetylglucose-6-iodohydrin, but of these only the first could be converted to the glucoseenide.

Müller has made use of the action of AgF on 6-halogenohexosides for the elucidation of ring structure. The resulting 5,6-enide was expected to reduce Fehling's solution directly in the case of furanosides and only after hydrolysis in the case of pyranosides. However, the author finds (41) that the AgF brings about desaturation only when the carbon adjacent to the terminal takes part in oxygen-bridge formation so that in the case of aldohexo-furanosides, desaturation does not take place. Hence, the reaction with AgF still retains its value for the purpose for which it was originally recommended.

By methylation of normal methyl riboside, Levene & Tipson (35) obtained the trimethyl derivative, and this on hydrolysis gave crystalline trimethyl ribose. Oxidation with bromine gave the lactone, having the characteristics of the δ -form. Oxidation with nitric acid gave a trimethoxy-glutaric acid, isolated as the ester. It was concluded that normal methyl riboside has a pyranose structure.

From *d*-glucoheptulose, Austin (17) has prepared the α -methyl glycoside and its pentacetate, and also *d*-glucoheptulose- α -hexacetate. From calculations of rotations it is concluded that all these derivatives are α -forms and contain the <2,5> ring. To the reviewers it

would seem that the conclusions are in need of chemical substantiation.

Levene, Raymond & Dillon (34) found 4-methylglucoheptose to be unlike 2- or 3-methylglucose in that it formed only the normal glucoside under conditions which led to both normal and γ - forms with the other sugars. Inasmuch as substitution in position 4 prevented γ -glycoside formation, it is concluded by analogy that the γ - glycosides of the unsubstituted sugars have the furanose ring.

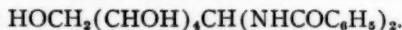
A preliminary note by Levene & Tipson, followed by a further paper by the same authors (36), describes the methylation of adenosine and hydrolysis of the methylated product to give a trimethyl ribose, different from 2,3,4-trimethyl ribose. Oxidation of the new product established it as 2,3,5-trimethyl ribose so that in adenosine the oxygen bridge of the sugar must have been of the furanose type.

Following the same method, these authors (37) have methylated guanosine and hydrolyzed the product. The trimethyl ribose obtained was identical with that from adenosine so that the ring is also of the furanose type in this nucleoside.

The X-ray work started previously by McCrea has been extended (38) to *d*-mannitol and *d*-mannose. Cell-size, space-group, and molecules per unit cell are determined for mannose, and it is concluded that the probable structure is that of a manno-pyranose.

A paper by Cox & Goodwin (21) is also concerned with a determination of ring structure from X-ray study of the ordinary and new gamma form of methylmannoside. It was concluded that of these the first was built up of pyranoside molecules arranged with the 6-atom ring parallel to the *c*-plane while the second contained molecules of the furanose type.

Open-chain sugars.—The problem of the non-cyclic glucose derivatives was investigated by Brügel, Mühlischlegel & Schinle (19). The special questions which these authors sought to answer were, first, whether it was possible to find conditions under which the cyclic form passes readily into the non-cyclic; and, second, to what extent it was possible to obtain non-cyclic derivatives of glucose by acting on bisulfite derivatives, cyanohydrins, and similar compounds. The authors first attempted to prepare non-cyclic aminoglucose by acting on aldehydo-glucose-pentabenzoylate with alcoholic ammonia. The resulting substance was, however,



They excluded the possibility that the substance was formed from an intermediate glucopyranose. The next attempt was to prepare a non-cyclic derivative from a partially substituted mercaptal. However, they failed to secure a substance of a lower degree of substitution than the tetrabenzyol mercaptal, and this on treatment with alcoholic ammonia gave the foregoing non-cyclic dibenzimide. Glucose, ammonia, and benzamide did not react to form this dibenzimide, but from 3,5,6-tribenzyolglucofuranose and methyl-alcoholic ammonia a small quantity of the dibenzimide was obtained. The authors concluded that for stability of the non-cyclic structure, the presence of acidic residues in the molecule is essential. Their efforts to obtain non-cyclic derivatives through the bisulfite derivatives or through the cyanohydrins were also unsuccessful. An interesting observation was made on 3,4,5,6-tetrabenzyoglucose. This non-cyclic derivative behaved as a true α -hydroxyaldehyde, undergoing ready polymerization and possessing no great tendency to form an ethylene-oxidic ring.

Continuing earlier work on the preparation and properties of the open-chain forms of the sugars, Wolfrom, Newlin & Stahly (52) report on the derivatives from xylose. Maltose-diethylmercaptal octacetate is also described.

Confirming the conclusions of previous papers, Wolfrom & Morgan (51) have studied the optical behavior of the hemiacetals of aldehydo-galactose pentacetate. Several of the hemiacetals are described, and the methyl derivative is further studied. In chloroform it gives a complex rotation curve but in methyl alcohol it has the form of a typical mutarotation. This would have been predicted from the theory previously proposed, which assumed three forms, *d*, *l*, and *aldehydo*.

The oxime of aldehydo-glucose pentacetate was previously studied. The acetylated aldehydogalactose oxime has now been examined by Wolfrom, Thompson & Georges (53) and compared with the ring form of the oxime acetate.

A preliminary note by Pacsu & Rich (45) suggests that α -pent-acetylfructose and α -chloroacetylfructose are both derivatives of the open-chain form of the sugar. The pentacetate was catalytically reduced, and the two alcohols which may theoretically be formed were both isolated. The chlorine is assumed to be attached to the first carbon atom for it is not replaced by any of the ordinary reagents. These conclusions are a confirmation of Hudson's deduction that the α - and β - forms in fructose are not a pair.

Pacsu (44) reached a similar conclusion in the case of turanose (glucosido-5-fructose) where the open-chain form is obtained along with others on direct acetylation of the sugar.

Orthoacetates.—In a paper on the third or “ γ ”-variety of triacetyl-methyl rhamnoside, Haworth, Hirst & Samuels (26) described an improved method for its preparation. The “ γ ”-monoacetyl compound was also prepared and methylated. The rate of hydrolysis of the methoxyl residue of the orthoacetate group was determined and the effect of acid upon the rates was studied.

In a paper on derivatives of turanose, Pacsu (44) describes a number of interesting isomers. Turanose had already been ascribed the structure of glucosido-5-fructose so that the furanose ring of the fructose residue was excluded. Despite this fact, four turanose octacetates were obtained. Pacsu considers these to be the normal octacetate, the open chain, and two isomers of the orthoacetate. The first octacetate is converted by HCl or HBr into the halogen derivatives, which are syrupy reactive products analogous to the fructose derivatives. The second gives no halogen derivative. Of the two orthoacetates, one is derived directly by acetylation of turanose and gives stable, crystalline halogen compounds. These in turn, with silver acetate, yield the fourth octacetate. Pacsu points out that in the formation of the orthoacetate a new asymmetric carbon atom has been created, and that the existence of two forms is therefore predictable.

Several derivatives of glucosido-4-mannose are described by Isbell (31). Reactions of the “ γ ”-glycoside indicate it to be of the ortho-ester structure. It forms a dextrorotatory chloro-compound on treatment with dry HCl in chloroform or methyl alcohol. It is noteworthy that the chloride in alcoholic solution, treated with dry silver carbonate, yields the β -methyl glycoside. If moist silver carbonate is used, a hexacetate is formed. The fact that the mannose residue is already substituted in position 4 excludes consideration of $<1,4>$ -ring forms.

Partially methylated sugars.—The methylated glucoses of Pacsu prepared from acetylated *d*-glucosidobenzylmercaptal were the subject of further discussion. Pacsu (43) himself and Schinle (48) have confirmed the finding of Levene, Meyer & Raymond that the substance to which Pacsu had assigned the structure of 4-methyl-glucose is in reality identical with the 2-methylglucose of Brügel. Later Schinle (49) discovered that the substance regarded by Pacsu

as 4,5,6-trimethylglucose is in reality a *monomethyl* glucose, probably 4-methylglucose.

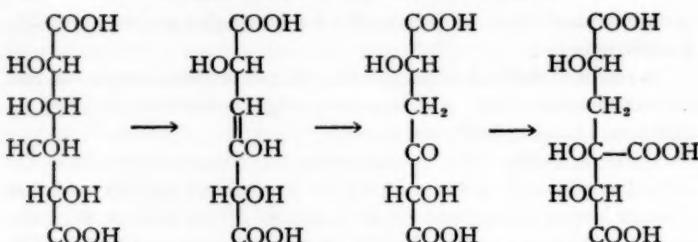
A modified method of preparation of 2, 3, 4-trimethylglucose and a determination of its structure by oxidation methods is given by Charlton, Haworth & Herbert (20).

Irvine & Stiller (30), re-examining the heptamethyl sucrose described by Haworth in his study of the structure of sucrose, find that it is not homogeneous, and in fact contains 27 per cent of the octamethyl derivative and less than 34 per cent of the heptamethyl. The latter, moreover, consists of a mixture of at least four isomerides. After hydrolysis of the methylated sucrose at least ten substances were obtained. The authors also suggested that during the methylation there may have been some change in the position of the ring of the glucose component. This was based on the isolation of a trimethyl derivative, presumably of glucose non-substituted in position 6, which, according to the authors, formed a γ -glucoside. Final evidence for the assumption as to the ring form has not as yet been furnished and the authors also consider the possibility that the product is a derivative of fructose and not of glucose.

Haworth, Hirst & Stacey (27) have methylated several monocarboxylic acids and lactones. Sodium- α -glucoheptonate, methylated first by the methyl-sulphate and then by the Purdie method, yielded the pentamethyl γ -lactone. Methylation of γ -galactono-lactone hydrate by the methyl-sulfate method gave a mixture of the tetramethyl- γ - and δ -lactone, while methylation of the anhydrous lactone by Purdie's method gave 2,3,6-trimethyl- γ -galactonolactone. Re-methylation with silver oxide and methyl iodide gave quantitatively the tetramethyl- γ -lactone. Methylation of the γ -lactones of arabonic and gluconic acids first by the methyl-sulfate and then by the silver-oxide method gave almost entirely the completely methylated derivatives.

On the basis of glycoside and lactone formation of the 5-methyl-glucose of Ohle & von Vargha, as well as on the properties of the triacetyl- β -methylglucosides, Levene & Raymond (33) conclude that the substance is in reality δ -methylglucose.

Miscellaneous.—In last year's review mention was made of a peculiar reaction of mannosaccharic acid with KCN leading to the formation of a tricarboxylic acid to which the composition $C_7H_{10}O_{10}$ was attributed. Kiliani now finds (32) that the acid has the composition $C_7H_{10}O_9$ and this fact permits an easy interpretation of the reaction, as follows:



To the dibenzoyl-mannitol of Einhorn & Hallandt, Ohle and co-workers assigned the 2,3- or 4,5- structure. Brügel & Grüner (18) disagree with these conclusions. The argument of Ohle was based upon the isolation, from the oxidation products of the dibenzoyl-mannitol, of a substance which he regarded as a dibenzoylmesotaric acid. Brügel and co-workers find that the substance is a benzoyl-glycolic acid, identical with the substance obtained by oxidation of hippuric acid with nitric acid.

Müller (42) has also studied this dibenzoylmannitol and he finds that the *p*-toluenesulfonyl derivative of the substance on treatment with NaI in acetone does not yield an iodine-containing substance. He therefore concludes that the benzoyl groups are attached to the primary alcoholic groups (1 and 6). This view is substantiated by the failure to prepare trityl derivatives of the dibenzoylmannitol. The monoacetone mannitol prepared through this dibenzoyl derivative is identical with the substance to which Irvine & Patterson have assigned the structure of 1,2-monoacetone mannitol. The author states that the 3,4-isopropylidenemannitol structure should be assigned to this substance. This conclusion is further substantiated by the formation of a ditrityl derivative of this monoacetone mannitol.

A brief note by Haworth (25) announces work on the hexuronic acid of Szent-Györgyi and suggests it to be probably the 6-carboxylic acid of a ketohexose. A more detailed account by Hirst & Reynolds (29) gives the properties of the acid and its *p*-bromophenylosazone and also describes oxidation experiments which indicate a reversible oxidation which is ascribed to formation of the osone.

Mentholglucuronic acid, obtained by feeding menthol to dogs, was found by Da Cruz (24) to be hydrolyzed by β -glucosidase, indicating that it is a β -glucoside. It is of interest to note that, although inactive menthol was administered, the conjugated menthol is optically active.

Revision of the properties of *d*-talonic acid are given by Cretcher & Renfrew (22) who report a slightly higher specific rotation than that of Hedenburg & Cretcher. The shape of the mutarotation curves indicates formation of a dextro-rotating δ -lactone as well as a levo-rotating γ -lactone. The latter was determined by titration and was also isolated in crystalline form.

III. REACTIONS

Migration and isomerization.—The problem of the migration of acetyl groups has been continued by Haworth, Hirst & Teece (65) who have methylated 2,3,4-triacetyl- α -methylglucoside and obtained 2-methyl-3,4,6-triacetyl- α -methylglucoside, thus having discovered a new example of the migration of an acetyl group in the course of methylation. The authors recommend caution in drawing structural conclusions based on the methylation of partially acetylated derivatives. They recall, however, the finding of Helferich & Günther that on methylation of 2,3,4-tribenzoylmethylglucoside, no rearrangement takes place.

A study of pentose reactions has been commenced by Hurd & Isenhour (66). This first paper deals with the conversion of xylose into furfural and the efficacy of various agents in promoting this change. The effect of mineral acids on a variety of substances is described and their relation to the mechanism of the pentose \rightarrow furfural reaction is discussed.

An isomerization phenomenon of mannosaccharic acid was observed by Rehorst (81). Kiliani previously described two potassium salts of mannosaccharic acid. One was obtained by the action of the alkali on the di-lactone and the other by boiling the diamide with KOH. The first reduced Fehling's solution or an alkaline solution of iodine; the second behaved normally as a dicarboxylic sugar acid. Rehorst has prepared the crystalline-mannosaccharic acid and finds that the salts of this acid do not reduce Fehling's solution or alkaline-iodine solutions, whereas the salts freshly prepared from the di-lactone do. On standing in aqueous solution the lactone is gradually hydrolyzed and the reducing power of the solution diminishes. Thus, the acid forming slowly from the lactone is the non-reducible isomer. The acid formed from the dibasic salt by neutralization differs from the crystalline variety in its rotatory power. The nature of the isomerism is not clear.

Dismutation.—The transmutations of trioses were further inves-

tigated by Fischer & Baer (62). Two substances, $\text{CH}_2\text{OH}-\text{CHOH}-\text{CHO}$ and $\text{CH}_3-\text{CHOH}-\text{CHO}$ were previously found to behave differently toward the action of pyridine at high temperatures. Whereas the first suffered transmutation to dihydroxyacetone, the second did not undergo the corresponding rearrangement into acetol. The present study deals with the behavior of 3-methyl-glyceraldehyde and it is found that it suffers a transformation analogous to that of unsubstituted glyceraldehyde and yields the methyl ether of dihydroxyacetone.

The action of calcium hydroxide upon various sugars has been studied by Austin, Smalley & Sankstone (56). It was found that, in the cases of xylose and arabinose, the process was largely irreversible, but that glucoheptose and glucoheptulose constituted a practically reversible system and the same end-point was obtained by using either of the pair as starting material. The conclusions are based largely upon optical data.

A preliminary report by Nicolet (75) describes the formation of α - β -diphenyl lactic acid in good yield by the action of alkali on $\text{C}_6\text{H}_5\text{CH}(\text{OCH}_3)\text{CHOHCOC}_6\text{H}_5$. The author explains this reaction as a benzilic-acid rearrangement of the diketone which is the intermediate product formed by an "aldol dehydration." Nicolet considers that Nef's interpretation of the mechanism of saccharinic-acid formation from sugars is in need of revision.

Continuing previous work on the alkaline degradation of carbohydrates, and its connection with carbohydrate oxidation, Evans & Hockett (59) have studied the yields of formic, acetic, and lactic acids and pyruvic aldehyde from the action of alkali on cellobiose, lactose, melibiose, and gentiobiose. The yields of the different products were in general agreement with the predictions made on the enediol theory previously proposed.

In similar experiments on 3-glucosidoarabinose, Evans & Clark (60) obtained results agreeing with the postulate that this substance is an intermediate in the alkaline degradation of the cellobiose and maltose.

Continuing work on the methylated sugars, Loder & Lewis (74) subjected 3-methylglucose to the action of dilute alkali and isolated as the principal product 3-methylfructose, identical with that prepared from α -diacetone fructose. Indirect evidence is given for the formation of a small amount of saccharinic acid and partially demethylated hexoses. At 35° the methyl group is removed slowly as

methyl alcohol, and at 100° the reaction is rapid. The absence of a high iodine absorption is taken as evidence that the 2,3-enediol does not form, the behavior resembling that of glucose rather than that of tetramethylglucose. Apparently only very small amounts of methylmannose could have formed.

Oxidation.—Papers have been published by Roepke & Ort (82) and by Ort (78) continuing previous work on the active reductant of glucose and other sugars, and the effect of temperature and concentration on the rate of formation of the active reductant has been measured for several of the commoner sugars. Ort concludes that the first step in the oxidation is the formation of the enediol and that only this form of the sugar undergoes oxidation. Experiments on glucose in alkaline solution, protected from air, show that hydrogen is the only reductant that accumulates to any extent within one or two days, its pressure being about 0.01 atmosphere.

From a study of the rotational changes resulting during oxidation of sugars by bromine water, Isbell & Hudson (70) conclude that the $\langle 1,5 \rangle$ -ring form of the sugar is directly oxidized to the δ -lactone and that this then hydrolyzes to the acid. Confirmation of this view is given by Isbell (67), who finds that barium carbonate reacts with the acid but not with the δ -lactone, so that if it were formed first the acid would be removed from the reaction. Actually this is not the case. Circumstantial evidence is presented to support the view that the aldoses exist in the $\langle 1,5 \rangle$ -ring form.

In a preliminary note on the oxidation of α - and β - forms of sugars by bromine water Isbell (68) reports that β -lactose is oxidized to the extent of 95 per cent in 3 minutes, while the α - form is oxidized to the extent of but 50 per cent in the same length of time. This affords a new method of ascertaining the approximate amounts of α - and β - isomers in solution. Mixtures exhibit breaks in the oxidation curves, so that this property will show whether ordinary sugars are α -, β - mixtures like the compounds reported by Hudson & Hockett. This problem is now being studied, as is also the relation between configuration and rate of oxidation.

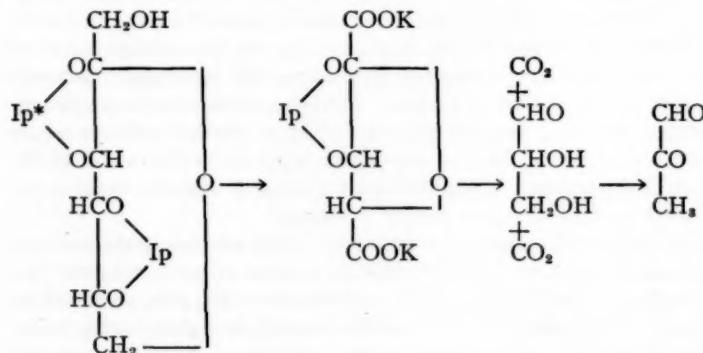
Oxidation of sugars in ammoniacal copper solution in the presence of air has been studied by Parrot in a series of papers. In the case of glucose (64), levulose (64), and mannose (80), there are formed oxalic acid, imidazole, and arabinose-tetrahydroxybutyl-4-imidazole. In the case of galactose (63), as expected, lyxose-tetrahydroxybutyl-4-imidazole was obtained instead of the arabinose derivative. The

oxidation of levulose by methylene blue (79) in ammoniacal solution was similar to the oxidation with copper.

The catalytic oxidation of sugars by oxygen in the presence of iron pyrophosphates has been studied by Degering & Upson (58) who have decided that the first step is an enolization.

According to Lal'Dube & Dhar (73), glucose inhibits the oxidation of insulin by air and is itself more rapidly oxidized than in the absence of the insulin. They consider this to be the mechanism of increased oxidation of glucose in the animal body in the presence of insulin. The authors mention, however, that putrefaction occurs and it would seem to the reviewers that this would introduce serious complications or even invalidate the conclusions.

In last year's review a reaction of Ohle & co-workers which was regarded by them as a model of the process of alcoholic fermentation was reported. Starting with β -diacetonefructose-1-sulfuric acid or β -diacetonefructose-1-phosphoric acid, Ohle & Garcia y Gonzalez obtained, on oxidation with $KMnO_4$, a dicarboxylic acid (branched chain). On hydrolysis this yielded CH_3COCHO along with other products. The same authors (77) now find that potassium diacetone-2-ketogluconate on treatment with $KMnO_4$ (equivalent to 9 atoms O) yields an analogous unstable acid, in this case tricarboxylic. The hydrolysis products of this acid are CO_2 , $CH_2(OH)COOH$, and $CH_2OH \cdot CHO$. The oxidation of β -diacetone fructose in initially neutral solution leads to a different set of reactions, which the authors formulated in the following way:



* Ip = Isopropylidene.

Ohle, Contsicos & García y Gonzalez (76) have reported on the oxidation of the derivatives of acetone glucose. Monoacetoneglucose-3-sulfuric acid and diacetoneglucose-3-sulfuric acid were oxidized in initially neutral solution and in each case monoacetonexyluronic-acid-3-sulfuric acid was the main product. In the case of diacetone glucose, carbon atom 3 was the most vulnerable and therefore monoacetonexyluronic acid was formed in small proportion. The saccharinic rearrangement which takes place in the case of β -diacetonefructose-3-sulfuric acid does not occur on oxidation of the above-mentioned glucose derivative.

Electrolytic oxidation of sugars to corresponding monocarboxylic acids has been advocated by Kiliani (71) for the preparation of *d*-galactonic acid.

Direct preparation of pure crystalline mannonic acid by hydrolysis of nut flour, followed by electrolytic oxidation, is described by Bernhauer & Irrgang (57).

A description of a semi-commercial preparation of calcium gluconate by electrolytic oxidation of glucose in the presence of calcium carbonate and a little calcium bromide is given by Isbell, Frush & Bates (59).

Oxidation of xylose to xylonic acid with chlorine in ammoniacal solution is described by Fang (61).

Oxidation of rhamnose to 6-keto- α -rhamnose is described by Votoček & Malachta (84).

A semi-industrial preparation of gluconic acid as the calcium salt, by fermentation of glucose with *Pencillium*, is described by Angeletti (55).

The mechanism of the Fenton reaction has been discussed by Küchlin (72) on the basis of the products of the catalyzed and non-catalyzed peroxide oxidation, and the kinetics of the reaction.

On heating a mixture of glutamic acid and glucose in glycerol, carbon dioxide is given off [Akabori (54)]. The sugar is believed to serve as oxidizing agent, the reaction being



In the first of a series of papers on oxidations induced by sugars, Shaffer & Harned (83) have studied the effect of different factors upon the yield of barium peroxide when barium hydroxide, glucose, and oxygen are shaken together. Up to 20 per cent of the absorbed

oxygen may be converted into barium peroxide. The primary product is considered to be a sugar peroxide, although no evidence is advanced to prove this to be the case.

IV. SYNTHESES

Partially substituted glucose.—A very promising starting material, 4,6-benzylidene glucose, was synthesized by Zervas (126) for the preparation of derivatives of glucose. Carbon atom 1 of the benzylidene glucose having acidic properties, the substance readily forms a sodium salt in which the sodium atom is readily substituted by alcohol or acid residues.

With a view to synthesizing derivatives of glucose substituted in position 4, Levene & Raymond (112) hydrolyzed the benzaldehyde residue from 2,3-dibenzoyl-4,6-benzylidene- β -methylglucoside and partially benzoylated the product to give 2,3,6-tribenzoyl- β -methylglucoside. The corresponding acetyl compounds were prepared in an analogous manner. Several derivatives are described and it is concluded that certain of them indicate that Josephson was mistaken regarding the structure of the 2,3-dibenzoyl glucose described by him.

New benzal and benzoyl derivatives of glucose have been prepared by Brügel & Grüner (88). The starting material for the benzoyl derivative was 4,6-benzylidene glucose. From this the authors obtained 1,2,3-tribenzoylglucose, 2,3-dibenzoyltriacetylglucose, 1,2,3-tribenzoyldiacetylglucose, and 1,2,3,6-tetrabenzoylglucose. The same authors prepared benzylidene-monoacetone glucose by treatment of monoacetone glucose with benzaldehyde, using $ZnCl_2$ as catalyst, and assigned to it the structure of 1,2-monoacetone-3,5-benzylidene glucose. The authors claim that the monoacetone-benzylidene glucose prepared by Levene & Meyer is identical with the one prepared by them, and that Levene & Meyer erroneously attributed to their substance the structure of 1,2-acetone-5,6-benzylidene glucose. The reviewers consider that the identity of the two has not been proven.

The action of boric acid and borates on the rotatory power of glucose, fructose, and galactose is reported by Darmois & Peyroux (90). Boric acid is without effect on glucose, but sodium borate forms complexes with all three sugars, two compounds being indicated in the case of levulose. The complexes exist only in alkaline solution.

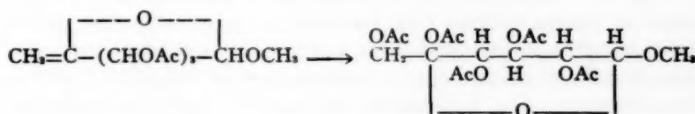
Brigl & Grüner (89) have introduced a new procedure for the preparation of partially acetylated sugars in which the di-esters of *o*-boric acid are first formed and then benzoylated without intermediate isolation. By merely boiling in water or in very dilute acid, the boric acid residues are removed. In the case of glucose, the product was found to be 2,6-dibenzoyl glucose. In the case of mannitol, it was 1,6-dibenzoylmannitol. The authors further conclude that the derivative considered by Josephson to be 2,3-dibenzoyl- β -methylglucoside is really the 2,6 isomer, a migration of a benzoyl group having occurred during the preparation.

Helferich & Bredereck (100) describe a method for separation of 2,3,6-triacetyl- β -methyl-*d*-glucoside from the 2,3,4 isomer. The method is based on the formation of the trityl derivative of the second isomer, which is practically insoluble in water. By extraction with water, the first isomer is obtained.

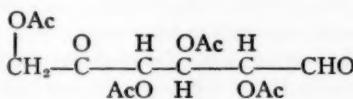
Partially methylated glucose.—Oldham & Rutherford (122) have prepared 2-methylglucose by a new method. They formed the γ -glucoside of 3,5,6-tribenzoyl glucose (from monoacetone glucose) and methylated it. Several derivatives are reported.

Irvine & Rutherford (104) converted 2,3-dimethyl-methylglucoside through the 4,6-dinitro derivative to the 4-nitro-6-iodo derivative. Treatment with silver acetate, followed by hydrolysis, methylation, reduction, and further hydrolysis, gives 2,3,6-trimethyl glucose.

Novel derivatives.—A new oxidation product of glucose was described by Helferich & Bigelow (99). The substance was obtained from triacetyl- β -methylglucosideenide through the action of $Pb(OAc)_4$. The pentacetate of a 5-keto- β -methyl glucoside, which the authors named pentacetyl- β -methylgluconoside, is thus obtained.



Hydrolysis with water gives tetracetylgluconose.



The acetate reduces Fehling's solution in the cold, gives Schiff's test, and forms a *bis-p*-nitrophenylhydrazone. Noteworthy is the fact that triacetyl- β -methyl-*d*-glucoside is reduced to triacetyl- β -methyl-*d*-isorhamnoside when the reduction is carried out in 95 per cent CH_3OH in the presence of Pd charcoal catalyst. However, in addition to this substance a sugar belonging to the *l*-series is also obtained when the reduction is performed in ether solution in the presence of Pt catalyst.

A novel modification of lactose prepared from α -lactose monohydrate in acid methyl alcohol is described by Hockett & Hudson (101). From its properties they conclude it to be a molecular compound of the form 5- α -lactose-3- β -lactose. The new compound is anhydrous. A similar substance has been secured by the same authors (102) in preparing methylxyloside and they believe it to be 7(α -methyl-*d*-xyloside) : 2-(β -methyl-*d*-xyloside), though the ratio may be 3:1. The molecular compound may be separated, though with difficulty, by crystallization from ethyl acetate.

Condensation of sugars with amino acids.—Much activity was displayed in the past year in the condensation products of sugars with amino acids. Of these substances two types have been prepared. In one the amino acids or peptides were linked in a glycosidic union with the sugar; in the other, through a peptide linkage with the amino group of glucosamine.

Among the publications dealing with the first group of substances there is to be mentioned that of Maurer & Schiedt (116) who describe the cellobiosides of several sarcosine derivatives. These glycosides, like ammonium glycosides and urea glycosides, are very unstable and readily reduce Fehling's solution.

In the second group is the tetracetyl-*N*-alanylglucosamine prepared by Bertho & Maier (86) by reduction of tetracetyl-*N*- α -azido-propionyl-glucosamine with PtO_2 catalyst. It was then converted into *N*-dialanyl-glucosamine by condensing with $\text{CH}_3\text{CHBrCOCl}$ in chloroform-pyridine, followed by treatment of the bromo-derivative with methyl-alcoholic ammonia.

N-Glycylglucosamine was prepared by the same method (87) through reduction of *N*-azidoacetylglucosamine in the presence of PtO_2 as catalyst. Incidentally it may be mentioned that β -azidoacetoglucose was reduced to β -aminoacetoglucose.

N-Glycyl-*d*-glucosamine and *N*-*d*-alanyl-glucosamine were prepared by Bergmann & Zervas (85) by action of carbobenzoylglycine-

chloride and carbobenzoyl-*l*-alaninechloride on tetracetyl- β -*d*-glucosamine. The authors came to the conclusion that the alanyl-glucosamine described by Bertho & Maier (86) was impure.

Oxidation of glycals.—Werner Freudenberg (93) prepared *d*-threose through xylal. The ozonide of diacetylxylose with zinc dust gave diacetyl-*d*-threose, and saponification of the latter led to the free threose.

Triacetylgalactal and perbenzoic acid have been shown by Levene & Tipson (113) to yield 1- β -benzoyl-3,4,6-triacetyl galactose in an analogous manner to the glucose derivative. Galactal, on the other hand, with perbenzoic acid gave a mixture of galactose and talose, the latter predominating.

In view of the report of Levene & Tipson on the preparation of *d*-talose from galactal, Komada (110) reported similar results. The talose was isolated in the form of its α -methyl-phenylhydrazone.

In a paper by Hurd & Isenhour (103) it is claimed that diacetyllyxosan or a mixture of it with diacetylxylosan is formed by the action of perbenzoic acid on diacetylxylose. The authors also describe certain derivatives of xylose, principally xylosan, formed by pyrolysis of xylose.

Glycosides.—Crystalline α -methylglucofuranoside has been prepared by Haworth, Porter & Waine (98) by acetylating the residues from β -methylglucofuranoside-5,6-monocarbonate followed by fractionation and hydrolysis. The crystalline glucofuranoside does not reduce Fehling's solution, nor is it hydrolyzed by zymin or emulsin. After even the gentlest acid treatment, Fehling's solution is reduced. Derivatives of both the α - and β -methylglucofuranosides are described.

From 3,4,6-triacetyl- β -glucosyl chloride and the silver salt of *p*-nitrophenol, Goebel, Babers & Avery (95) have prepared the α - and β -*p*-nitrophenol glucosides. These are of interest from the chemical standpoint because of the fact that in the presence of OH ions they are unstable and apparently interconvertible.

The synthesis of guaiacol glucoside from acetobromoglucose and guaiacol in quinoline is described by Kariyone & Horino (108).

The separation of mixtures of the methyl-*d*-gulosides into the crystalline components is again described by Isbell (106). A number of new derivatives, including the acetates, are reported.

Continuing work on derivatives of fucose, Minsaas (118) has prepared the crystalline α - and β -methylfucosides.

From commercial polygalacturonide by treatment with methyl alcohol and dry HCl, Link (114) has prepared methyl galacturonide methyl ester, and through the barium salt, the crystalline methyl-*d*-galacturonide dihydrate.

Di-, tri-, and tetrasaccharides.—A new synthesis of cellobiose was reported by Freudenberg & Nagai (91) by treatment of 1,6-levo-glucosan with acetobromoglucose in dioxan solution. The product was treated in the cold with 50 per cent sulfuric acid in order to open the <1,6> ring (which is less resistant than the disaccharide linkage). After removal of the sulfuric acid, the product was acetylated, and from the product acetylcellobiose was obtained in small yield.

From 3,4,6-triacetylglucose-1,2-anhydride and 2,3,4,6-tetracetylglucose, Haworth & Hickinbottom (96) have prepared a new disaccharide, isomeric with trehalose and named by them "neotrehalose." It is considered to be α -glucosidyl- β -glucoside. Its heptacetate and octacetate are described. The substance does not have the rotation predicted by Hudson's rule.

Continuing earlier attempts to synthesize sucrose, Irvine & Stiller (105) have devised new methods for the preparation of the tetracetylglucose and tetracetylfructofuranose used as starting materials. Several variations for condensation were tried, including an exact repetition of the conditions of Pictet & Vogel, but in no case was sucrose octacetate obtained. Crystalline isosucrose and isotrehalose and glucose acetates were isolated, as well as non-crystalline mixtures containing a variety of acetylated diglucoses and difructoses and at least one additional glucofructose.

A new maltose anhydride was prepared in crystalline form by Karrer & Kamienski (109) by heating 1-dimethylamino-heptacetyl-maltose with an aqueous solution of barium hydroxide.

Freudenberg & Nagai (92) described the synthesis of decamethyl- β -methylcellotrioside by condensation of the 1-chlorohydrin of heptamethylcellobiose with 2,3,6-trimethyl- β -methylglucoside. A complex mixture was obtained, from which the methylated cellotrioside was isolated with difficulty.

The application of mercury salts as condensation agents has been further extended by Zemplén & Gerecs (125). Tridecacetyl-1- β -methyl-6'- β -cellobiosidogentiotiobiose was obtained by condensation of aceto-6'- β -cellobiosidoglucose with 1- β -methyltriacetylglucose.

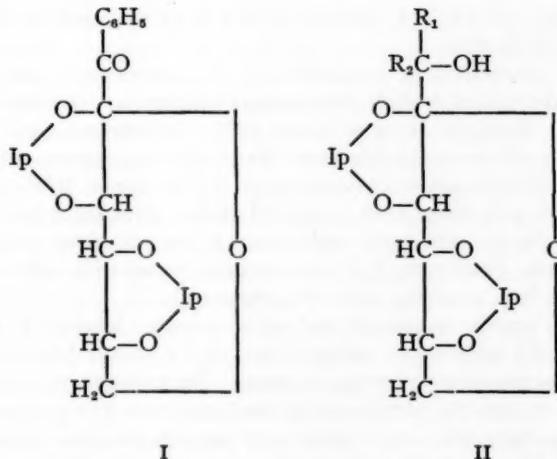
Uronic acids.—The hitherto unknown *d*-mannuronic-acid lactone

was isolated by Nelson & Cretcher (119) from the alginic acid of *Laminaria saccharina* and *Fucus serratus*.

The preparation of *d*-mannuronic-acid lactone from alginic acid is also described by Schoeffel & Link (123), the procedure being similar to that used by them for the preparation of galacturonic acid. The properties reported for mannuronic-acid lactone by Nelson & Cretcher (119) are confirmed.

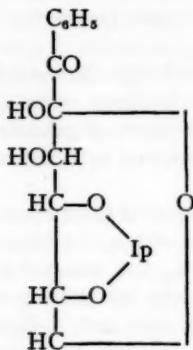
Exact directions for the preparation of crystalline *d*-galacturonic acid from a commercial polygalacturonide from citrus pectin are given by Link & Nedden (115). Synthesis of *dl*-galacturonic acid by reduction of mucic-acid lactone is described by Niemann & Link (120).

Miscellaneous. — Starting with diacetone-2-ketogluconic acid or its methyl ester, Ohle & Blell (121) prepared, by the Grignard reaction, sugar derivatives containing a phenyl group attached to the first carbon atom. When the diacetone acid was the starting material a mixture of diacetone-1-phenylglucosone (I) and β -diacetone-1,1-diphenylfructose (II) was obtained, but the methyl ester gave only the derivative of fructose. With benzyl magnesium chloride, diacetone-1,1-dibenzylfructose is formed.

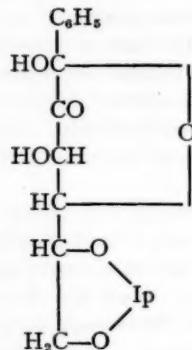


Ip = Isopropylidene

On deacetonation of I, a crystalline 1-phenylglucosone was obtained. This is the first crystalline osone reported. On partial deacetonation of I, two monoacetone derivatives were obtained, to which the authors assigned the following structures:



III



IV

Ip = Isopropylidene

Thus, the authors think that the reaction is accompanied in part by a shift of the ring.

The preparation of α -glucoheptose, its pentamethyl and hexamethyl derivatives, and the pentamethyl- δ -lactone are described in a paper by Haworth, Hirst & Stacey (97). Acetobromo- α -glucoheptose with old silver chloride gives the α -chloro compound, whereas fresh, activated silver chloride gives the β - form. The bromo-compound with silver oxide in methyl alcohol gives the β -glucoside, whereas the β -chloro compound treated in the same way yields the α -glucoside. Finally, the β -chloro compound heated with methyl alcohol and a little quinoline gives the orthoacetate.

From acetobromoglucose and silver cyanate, Johnson & Bergmann (107) isolated two isomeric tetracyetyl-*d*-glucose-*l*-isocyanates, as well as *sym*-octacetyl-*d*-diglucoseurea. The isocyanates react with alcohols to give the corresponding urethanes, and it is proposed to condense them with amino acids and peptides to form glycosidoureides. Silver cyanide and acetobromoglucose give the crystalline isonitrile on which further work will be done.

The synthesis of *d*-threose from xylose by Wohl's method has been described by Mendive (117).

A series of new thioes (from xylose, arabinose, glyceric aldehyde, and glycolic aldehyde) were prepared by Gehrke & Kohler (94).

Preparation of the acetates of several monobasic-sugar-acid lactones has been described by Upson & Bartz (124). They conclude that Mikšič's compound was not tetracetyl- γ -gluconolactone, but the acid.

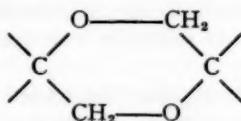
From divinyl glycol with silver chlorate and OsO₄, Lespieau & Wiemann (111) have prepared racemic mannitol more easily than by the customary synthesis through acrolein or glycerol.

V. POLYSACCHARIDES

It is not possible, within the limits of this review, to give an adequate account of the recent work on polysaccharides. Speaking broadly, it seems to the reviewers that the general problem is still rather in a state of discussion, for but few of the conclusions are accepted generally and without reservation. The present review will be limited to a discussion of the work dealing with structure. The purely chemical methods employed for the solution of the problem may be classified in four groups: (1) thermal dissociation; (2) acetolysis; (3) partial hydrolysis of the natural products or of the methylated products; (4) complete hydrolysis of the methylated products.

Inulin.—Irvine (151) reported that trimethylinulin on hydrolysis gave rise, in addition to trimethyl- γ -fructose, to ω -methoxy-5-methylfurfural, tetramethyl- γ -fructose, and a trimethylanhydrofructose. The yield of the tetramethyl- γ -fructose varied from 2.7 to 1.7 per cent, and, on the basis of these yields, the chain could be calculated to contain 25 to 43 fructose units. This author seems to accept the chain structure only with reservations. Haworth, on the other hand, is emphatic in sponsoring the theory of the chain structure. Haworth & Streight (146) have prepared trimethylinulin by an improved method, and Haworth, Hirst & Percival (138) hydrolyzed it and obtained principally 3,4,6-trimethylfructofuranose. In addition, 3.7 per cent of 1,3,4,6-tetramethylfructofuranose was obtained. Assuming that the latter substance was the end member of the methylated polysaccharide, a chain of 30 units of fructofuranose residues or a molecular weight of 5,000 was suggested for inulin.

In disagreement with this view stood the observations of earlier workers to the effect that inulin on hydrolysis yielded a fraction which differed from the rest of the molecule in its resistance to hydrolysis. Jackson & McDonald (153) had reported the isolation from this fraction of a non-reducing difructoseanhydride and they later described (154) the isolation of two additional crystalline anhydrides. These findings were offered by them as evidence in favor of the presence in inulin of components of varying structure. However, Schlubach & Elsner (159) found that on acetylation, followed by permethylation and hydrolysis of the resistant anhydride, there was formed 3,4,6-trimethylfructose identical with that previously obtained by Haworth & Learner on hydrolysis of trimethyl inulin. Moreover, Haworth & Streight (147) prepared one of the three isomers and found that on acetylation, followed by methylation and hydrolysis, it gave the 3,4,6-trimethylfructofuranose identical with that obtained by Haworth & Learner on hydrolysis of trimethylinulin. They concluded that the remaining two anhydrides represented the stereoisomers of the one prepared by them, inasmuch as three isomers, $\alpha\alpha'$, $\alpha\beta$, and $\beta\beta'$, are theoretically possible. Haworth (138) explains the stability of these anhydrides on the assumption of the dioxan character of the anhydride ring:



All these anhydrides are regarded by the authors as secondary products of hydrolysis.

Criticism of the older work on depolymerization of inulin is presented by Berner (131) who finds that the molecular weight estimations of "inulans" made in recent years were incorrect inasmuch as the substances were generally contaminated with the solvent used in the reaction.

Pringsheim & Hensel (156) have published an interesting communication on the enzymatic hydrolysis of inulin. Of 15.0 gm. of inulin, 0.5 gm. remained unhydrolyzed, the unhydrolyzed product being different from the stable difructose anhydride of Jackson & co-workers. On the other hand, Weidenhagen (163) reported com-

plete cleavage of inulin into fructose by means of β -fructofuranosidase obtained from yeast by the tannin method.

Irvine & McGlynn (152) advanced experimental evidence to the generally accepted view that the isolation of 2,3,6-trimethylglucose from a permethylated di- or polysaccharide does not give complete information as to the place of linkage between the individual glucose residues, inasmuch as either position 4 or 5 could have served as the place of union.

In conclusion, mention must be made of "levan," a synthetic isomer of inulin, obtained by Hibbert, Tipson & Brauns (149) by the action of *B. mesentericus* on sucrose. The substance was previously obtained by Greig-Smith and named "gum levan." The present authors showed it to be a polymerized anhydrofructofuranose. The polysaccharide differs from inulin in that the hydroxyl groups involved in the linkages are located in positions 2 and 6, whereas in inulin they are located in positions 1 and 2.

Starch and glycogen.—Most of the contributions on the structure of starch and glycogen during the past year come from the sponsors of the theory of the straight-chain structure of these polysaccharides. Freudenberg, Werner Kuhn, and their associates (136) based their conclusions on the kinetics of the hydrolysis of the polysaccharide. The theory received additional support from the observations of Freudenberg and his associates (135) on the optical rotations of methylated trioses or tetraoses. These agreed with the deductions made on the assumptions of uniform linkage and of the validity of the superposition theory and the rule of distance. The methylated trioses and tetraoses were purified by distillation and were submitted as evidence of the chain structure of the polysaccharide.

Haworth & Percival (143) degraded the methylated polysaccharides by means of acetyl bromide and obtained methylated di-, tri-, and tetrasaccharides from the cleavage products. They offered these results as evidence of the chain structure of starch and glycogen. Later Haworth & Percival (144) hydrolyzed permethylated glycogen and found, in addition to the 2,3,6-glucopyranose, 9 per cent of 2,3,4,6-tetramethylpyranose. They therefore concluded that glycogen consisted of a chain of not more than 12 glucose residues.

Hirst and his associates (150) concluded from similar evidence that amylose and amylopectin have the same chemical structure. The differences in the substances are assumed to be due to differences in their micellar structure.

Irvine (151) has again warned against attaching too great a value to the isolation of tetramethylglucose as a basis for the formulation of the structure of starch. The findings in his own laboratory went to show that the character and the ratios of the different partially methylated glucoses and of tetramethylglucose differed, depending upon whether native or altered material was employed for methylation. Thus when amylose was methylated directly, from 3 to 5 per cent of tetramethylglucose was obtained. However, a special fraction of amylose gave, on hydrolysis, the following products: 2,3,4,6-tetramethylglucose, 23 to 26 per cent; 2,3,6-tetramethylglucose, 55 to 52 per cent; 2,3- and 2,6-dimethylglucose, 21 per cent, the two latter occurring in about equal proportion. On the other hand, in the case of acetylated amylose, the ratio is 1:3.

New polyamyloses, which shed little additional light on the structure of starch, were described by Pringsheim and co-workers (157).

A criticism of the work on the identity of hexosan was presented by Berner & Petersen (132). The tri- and the hexa-hexosans which were obtained by Pictet and collaborators through heating of starch in glycerol, glycol, and acetamide are, according to these authors, in reality substances of much higher molecular weight. The low molecular weight previously found was due to contamination of the substances by glycerol and alcohol. The "monohexosan" seems to be a condensation product of a hexosan with glycerol.

In view of the uncertainty of the relationship between the α -diamylose and α -tetra-amyllose (Schardinger's α -dextrin), Ullmann, Trogus & Hess (162) have undertaken a Roentgenographic study of these substances and have found that the X-ray pictures of α -amyllose varied greatly, depending upon the method of preparation.

Cellulose.—The greater number of contributions on this subject during the last two years advocated the straight-chain structure for the cellulose molecule.

Zechmeister & Tóth (166) advanced new evidence to this effect based on the older method of partial hydrolysis introduced by Bertrand & Benoist (133) and by Willstätter & Zechmeister (164). Following hydrolysis of cellulose with strong hydrochloric acid the present authors isolated several polysaccharides—cellotriose, $C_{18}H_{32}O_{16}$; cellotetraose, $C_{24}H_{42}O_{21}$; and a substance which corresponded to cellobhexaose, $C_{36}H_{62}O_{31}$. The cellotriose was found to be practically identical with the procellose of Bertrand & Benoist (133). The authors regard this finding as definite evidence in

favor of a gradual hydrolysis of a long chain composed of glucose residues. The occurrence of an isocellobiose among the products of hydrolysis could not be confirmed. The formation of Bertrand's procellose through the acetolysis of cellulose was also confirmed by Haworth, Hirst & Ant-Wuorinen (137), and these authors likewise deny the formation of an isocellobiose.

Freudenberg, Werner Kuhn, and associates (136) have applied the methods referred to in connection with the structure of starch to the problem of cellulose and adduce evidence for the straight-chain structure of cellulose.

Haworth, Hirst & Thomas (139) improved the process of methylation of cellulose and Haworth & Machemer (141) subjected the methylated cellulose to complete and to partial hydrolysis. Complete hydrolysis led to 2,3,6-trimethylglucose and to 0.6 per cent of 2,3,4,6-tetramethylglucose. From these data and on the assumption that the tetramethylglucose represents the end member of the chain, they conclude that the cellulose molecule is composed of a uniform chain of β -glucose residues and has a molecular weight in the neighborhood of 30,000. On similar grounds they have attributed (142) to cello-dextrin a molecular weight of approximately 3,000 to 4,000. The structure is accepted as being identical with that of cellulose.

On partial hydrolysis of completely methylated cellulose, Haworth and co-workers (140) have obtained a decamethylcellotriose and possibly a tridecamethylcellotetraose. This finding confirms that of Freudenberg. Again the authors offer this finding as evidence in favor of the chain structure of cellulose.

On the other side of the question stand the investigations from the laboratories of Irvine and of Hess. Irvine himself and his co-worker, Bell, have warned against final conclusions based on hydrolysis of methylated polysaccharides. Irvine particularly places little reliance on observations on polysaccharides which have been acetylated prior to methylation, inasmuch as in the course of this reaction a depolymerization of the polysaccharide may take place. Indeed, on hydrolysis of the permethylated native cellulose, Bell (127) could not detect even traces of tetramethylglucose, whereas the substance was obtained to the extent of 0.5 per cent (approximately the same proportion as found by Haworth and his co-workers) in the case of acetylated cellulose.

The validity of the mathematical argument of Werner Kuhn and Freudenberg in favor of the chain structure was questioned by

Klages (155), who developed a general equation of the progress of hydrolysis based on the assumption that the end member of the chain (having the aldehydic-sugar group) is labile. The author comes to the conclusion that the rate of hydrolysis could not serve as a basis for determining the structure of polysaccharides.

Bell (127), working under the direction of Irvine, found that wood cellulose differed in its properties, depending upon its source. Moreover, all forms of wood cellulose contained portions which were more resistant to hydrolysis (128) and these "resistant portions" differed in celluloses of different origins (129).

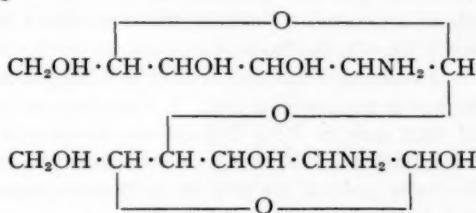
The communications from the laboratory of Hess dealt with the problem of the homogeneity of the celotrioses of Bertrand, Willstätter & Zechmeister, etc. Ullmann & Hess (161), on one hand, found that the values obtained for the molecular-weight estimation of the substance varied with the concentration, the lowest being 223 and the highest 964. Dziengel, Trogus & Hess (134) found that the substance could be separated into two fractions, one having the properties of a cellulose hydrate and the other a celotriose. According to the authors, this finding eliminates the celotriose as evidence in favor of the straight-chain structure for cellulose.

Communications contributing little to the question of the structure of cellulose but nevertheless of general interest for cellulose chemists are those by Wood (165) on cellulose methylene ether and by Traube, Blaser & Lindemann (160) on the sulfuric-acid esters of cellulose. The action of sodium on cellulose in liquid ammonia was described by Scherer & Hussey (158). The cellulose combines with 3 atoms of sodium per glucose unit, the first equivalent acting with greater velocity.

Finally, mention must be made of the so-called "synthetic cellulose" of Hibbert and co-workers. This cellulose is obtained by the action of *Acetobacter xylinus* on glucose, fructose, sucrose, mannitol, glycerol, and glyceraldehyde. The substance obtained by the action of the micro-organism on glucose is considered by the authors (148) to be identical with cellulose. On permethylation followed by hydrolysis, 2,3,6-trimethylglucose was obtained.

Chitin.—The structure of chitin has been the subject of several investigations. On acid hydrolysis of chitin, Bergmann, Zervas & Silberweit (130) obtained a disaccharide. This gave an octacetate of which two acyl groups were more resistant to hydrolysis than the others. These two were assumed to be located on the nitrogen atoms

and the conclusion was reached that the nitrogen atoms did not take part in the glycosidic union between the two components. If the union had been through the nitrogen atom the disaccharide should have combined with nine acetyl groups and, on treatment with alkali, should have lost eight acetyl groups (one from the C-N-C-group), thus leading to a monoacetylated derivative. From the fact that chitobionic acid on acetylation forms only one double bond between carbon atoms 2 and 3, whereas glucosaminic acid on similar treatment forms a doubly unsaturated acid (2,3, and 4,5), the authors came to the further conclusion that the union between the two glucosamine residues takes place in position 4. Hence, the structure of the biose is



They conclude further that chitin is a long chain of *N*-acetylated chitosaminic residues.

Zechmeister & Tóth (167) hydrolyzed chitin with cold hydrochloric acid and, on acetylation of the reaction product, obtained *N*-acetylglucosamine, the biose acetate described above, and chitotriose undeca-acetate (168), thus confirming the conclusion of Bergmann and co-workers regarding the structure of chitin.

Xylan.—The ring structure of the xylose units entering into the structure of xylan was the subject of a communication by Haworth & Percival (145). Dimethylxylan was hydrolyzed in acetic anhydride containing 1.6 per cent of sulfuric acid, and the product of hydrolysis was oxidized with bromine. On methylation, the methylester of hexamethyldixylobionic acid was obtained. Hydrolysis of this acid gave 2,3,4-trimethylxylopyranose and 2,3,5-trimethyl- γ -xylonolactone. The authors conclude that the xylose residue has the pyranose structure.

VI. NATURAL PRODUCTS

The sugar of a naturally occurring nucleoside, crotonoside, has been identified by Cherbuliez & Bernhard (169) as *d*-ribose.

Fucose, and a second hexose which could not be isolated in a pure state, have been obtained by Colin & Ricard (170) from *Pelvetia canaliculata*.

The sugar, sarmentose, from sarmentocymarin, has been obtained in crystalline form from the glycoside by Jacobs & Bigelow (172). It is a methyl ether α -desoxy hexose, isomeric with cymarose.

The preparation of quinovose from cinchona bark and its identification as isorhodose was described by Votocek & Rác (175).

An improved procedure has been described by Rimington (174) for the isolation of the carbohydrate complex of serum proteins. The structure glucosamine-dimannose is assigned, instead of the original structure of glucosamine-mannose.

The mode of occurrence of alginic acid is described by Dillon & McGuinness (171). On the basis of analysis, molecular weight, and acid value, it is concluded that alginic acid is a lactone and that the polymerizing unit is the complete acid. A formula is proposed.

The acid ester isolated from *Macrocystis pyrifera* is concluded by Nelson & Cretcher (173) to be a methylpentose monosulfate polymer. Fucose is probably the only methylpentose constituent.

VII. PHOSPHORIC ESTERS

The papers reviewed under this heading are confined to those which deal with structure or preparation, inasmuch as the biological functions of this group are dealt with elsewhere in this volume.

Various salts of fructose diphosphate have been described by Neuberg & Scheuer (181), but of these only the alkaloidal salts are easily crystallizable. The benzidine salt, remarkably, contains three mols of base to one of acid.

The synthesis of glyceraldehyde-3-phosphoric acid from glyceraldehydemethylcycloacetal was unsuccessfully tried by Fischer & Baer, and was finally achieved (177) by using glyceraldehyde-benzylcycloacetal. They were unable to prepare the acid or neutral barium salt, using barium hydroxide, as the product was destroyed by the alkali. A later paper by Fischer (176) describes the preparation of the crystalline calcium salt, using calcium acetate followed by alcoholic precipitation.

Galactose was phosphorylated in position 6 by Levene & Raymond (180) with the object of investigating the possibility of thus inducing fermentation of galactose by ordinary yeast. The starting

material for the synthesis was diacetone-*d*-galactose. The identical product was prepared by the same method by Hvistendahl (178), but due to intermediate isolation of the diacetone phosphate, his yield was lower than that of the former authors. The rotations reported by the two sets of workers agreed well.

The structure of the ribosephosphoric acid obtained from yeast nucleotides was studied by Levene & Harris (179), who succeeded in preparing it by hydrolysis of xanthyllic acid. It differs from the ribosephosphoric acid from inosinic acid in the rate of hydrolysis of the phosphoric acid residue and in showing different optical rotations during lactone formation. On the basis of these properties it was concluded that in the ribosephosphoric acid from xanthyllic acid the phosphoric acid residue is in either position 2 or 3.

The structure of the nucleotides is discussed elsewhere in the present volume (pp. 109 ff.).

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THE CHEMISTRY OF THE AMINO ACIDS AND THE PROTEINS*

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During the past year no hitherto undescribed amino acids have been isolated from proteins.¹ It was pointed out in last year's review (1) that a considerable number of cleavage products of the protein molecule have been described which, however, have to date lacked confirmation and have not yet conformed to the criteria which were laid down for an "accepted" amino acid by Vickery & Schmidt (2). In view of the fact that many of the analyses of the amino acid content of proteins do not account for all of the nitrogen present, it is to be expected that not all of the building stones which constitute the protein molecule have as yet been recognized. It must also be borne in mind that a part of the unaccounted nitrogen in proteins may result from the inadequacy of, and inherent errors in, our analytical methods. In view of the comparatively crude analytical methods upon which the estimation of the amino acid content of proteins is based, it is most surprising, if not astounding, that the literature contains so much data which, judging from the number of figures reported, point to a degree of accuracy far in excess of the reliability of the method. A like criticism can of course be directed at many of the other data which have been published in biological literature.

With a view to checking the occurrence of norleucine as a constituent of certain proteins, Czarnetzky & Schmidt (3) prepared this amino acid by hydrolyzing beef spinal cord.² A crude fraction containing the isomeric leucines, valine and tyrosine, was obtained by direct crystallization. Fractionation of the copper salts was followed by fractionation of the zinc salts according to the technique of Brazier (4). The product was freed from tyrosine by solution in hot glacial acetic acid and the isomeric leucines were fractionated by

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¹ Since this was written, M. Wada [*Biochem. Z.*, **257**, 1 (1933)] has announced the isolation of citrulline from a tryptic digest of casein. On heating with acid, citrulline is converted into proline.

² E. Abderhalden & K. Heyns [*Z. physiol. Chem.*, **214**, 262 (1933)] have again isolated norleucine from nerve substance. See also T. Yaginuma, G. Arai & K. Hayakawa, *Proc. Imp. Acad. Tokyo*, **8**, 91 (1932).

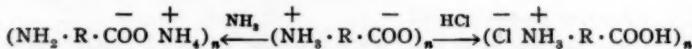
differential solubility in water. The tenth fraction contained pure norleucine. In order to characterize the amino acid further, Czarnetzky & Schmidt (3) measured the dissociation pressures of the three isomeric leucines when treated in the dry state with dry ammonia or with dry hydrogen chloride. At 40° the dissociation pressure of the *dl*-leucine-ammonia compound was 16.95 mm., that of *d*-norleucine 18.40 mm., and that of *dl*-isoleucine 17.55 mm. The dissociation pressure of the norleucine isolated from spinal cord protein was identical with that of the synthetic compound. The evidence at hand is now sufficient to warrant the inclusion of norleucine in the list of the "accepted" amino acids. Czarnetzky & Schmidt have used the method of titrating mixtures of the isomeric leucines in the dry state with ammonia gas to estimate the amount of each amino acid present in the mixture. By measuring the ammonia and the hydrogen chloride dissociation pressures at 0°, 25°, and 40° respectively of the isomeric leucines, they obtained the thermodynamic data which are given in Table I. Their experiments point to the existence of the amino acids

TABLE I

	<i>dl</i> -Leucine	<i>d</i> -Norleucine	<i>dl</i> -Isoleucine
pK _b * (25°)	1.90	1.86	1.88
pK _a * (25°)	2.45	2.44	2.44
ΔHK _b * (calories)	7,050	7,140	6,800
ΔHK _a * (calories)	2,840	2,820	2,900
ΔFK _b * (calories)	2,590	2,550	2,560
ΔFK _a * (calories)	3,340	3,330	3,330
ΔSK _b * (calories per degree)....	14.96	15.39	14.22
ΔSK _a * (calories per degree)....	-1.68	-1.71	-1.44

NOTE.—The terms, pK_a* and pK_b*, are defined as the negative (common) logarithms of the dissociation pressure or fugacity in atmospheres of a compound existing in the solid state. The values for pK_b* are for the combination with ammonia. The values for pK_a* are for the combination with hydrogen chloride.

in the dry state almost wholly as "Zwitter Ionen." The combination between the dry amino acid and gaseous ammonia and hydrogen chloride can be represented by the schematic equation:



The dissociation pressures of the ammonia and the hydrogen chloride compounds of the other naturally occurring amino acids have been determined by Czarnetzky & Schmidt. These will be published in the near future.

Abderhalden & Beckmann (5) have sought to differentiate the three isomeric leucines by converting them into the brom derivatives and following the rate of reconversion of the latter products into the corresponding amino acids, a procedure which had previously been used to differentiate valine from norvaline (6), but found very little difference in the rates. A more marked difference was noted when the brom derivatives were treated with methylamine and converted into the betaines. Further evidence of the occurrence of norvaline in proteins has been furnished by Abderhalden & Heyns (7) who report its occurrence in steer horn. Methionine and histidine were likewise present. The yield of methionine was 1 gram from 5 kilos of horn. These workers found that valine differs from norvaline in that only the latter undergoes a Walden inversion when converted into the brom acid and then back into the amino acid by treatment with ammonia. The hydantoins of these two amino acids have different melting-points. It is highly desirable that the occurrence of norvaline in proteins be confirmed by another laboratory and by the use of other criteria.

Emerson & Schmidt (8) have carefully re-examined the evidence which Van Slyke & Hiller (9) and Van Slyke & Robson (10) have brought forth in favor of the existence of dihydroxypyrrrole alanine as a constituent of gelatin and have failed to confirm its presence. Their work points to the possibility that the described product was a mixture of several of the known amino acids. Dakin (11) reports the synthesis of β -amino-*n*-valeric acid by treating propylidene acetic acid with ammonia under pressure.

Pirie (12) has improved the method of isolating methionine by the use of butyl alcohol. He obtained a yield of 1.4 per cent from casein. Du Vigneaud & Meyer (13) were able to isolate methionine from an enzymatic casein digest. Harington & Randall (14) synthesized β -hydroxyglutamic acid by treating ethyl-acetone-dicarboxylate with ethyl nitrite and hydrochloric acid, which yielded ethyl- α -iso-nitroso-acetone-dicarboxylate. This was reduced by hydrogen in the presence of palladium black and the resulting product boiled with concentrated hydrochloric acid. The inactive amino acid crystallizes with 3 molecules of water. The hydrated acid melts at 75°, resolidifies, and decomposes at 185°. The amino acid contains two asymmetric carbon atoms. It was not resolved. A confirmation of Dakin's work on the preparation of the naturally occurring β -hydroxyglutamic acid in the crystalline state has not yet been reported.

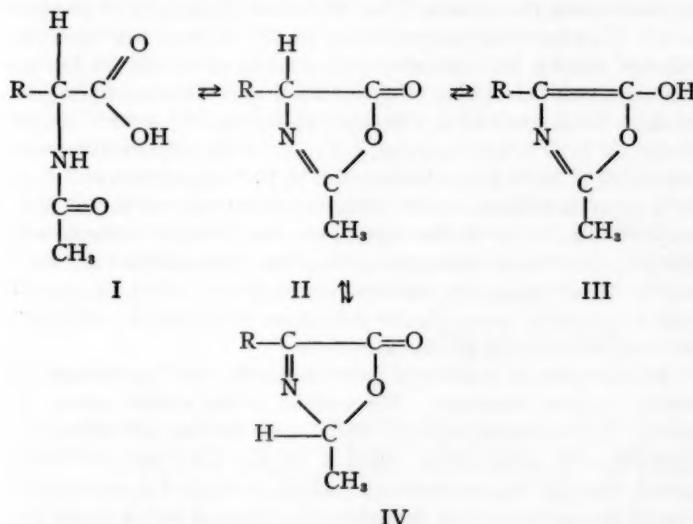
For the purpose of analysis it is highly desirable that quantitative

methods for the estimation of amino acids be available. Several new procedures have been recently reported. Leland & Foster (15) estimate thyroxine in the thyroid gland by extracting a solution made alkaline with sodium hydroxide with butyl alcohol. The thyroxine-distribution ratio between butyl alcohol and sodium hydroxide solution is 92:8, while diiodotyrosine distributes itself in the ratio of 2:98. The estimation is made on the basis of the iodine content. The method for estimating phenylalanine in proteins has been in a particularly unsatisfactory state. Kapeller-Adler (16) estimates it colorimetrically by nitrating the phenylalanine to 3,4-dinitrobenzoic acid and converting the latter product by means of hydroxylamine in the presence of ammonia into the bluish-violet ammonium salt of *o*-diacidihydro-dinitrobenzoic acid. Baernstein's (17) method for estimating methionine in proteins is based on the fact that the methylthiol group of methionine reacts with hydriodic acid in a manner similar to that of a methoxy group. The assumption which appears to be supported by fairly good evidence is made that proteins do not contain the methoxyl or methylimide groups. No other amino acid was found to yield a volatile iodide when treated with hydriodic acid. Analysis (18) of a considerable number of proteins shows that cystine (and cysteine) and methionine account for all of the sulfur present. However, in a number of instances, the difference is sufficiently large so that the possibility of the presence of another sulfur containing amino acid is not entirely excluded. The percentage content of methionine in the more common proteins is: casein, 3.5; fibrin, 2.4; edestin, 2.1; gliadin, 2.0; lactalbumin, 2.6; zein, 2.4; arachin, 0.5. Fürth & Minnebeck (19) estimate proline and oxyproline in proteins by a combination of Van Slyke's amino nitrogen technique, Dakin's butyl alcohol extraction method, and Kapfhammer's technique, which involves precipitation by cadmium chloride. They report the following percentage content of prolin: gelatin, 0.9; zein, 8.4; and casein, 5.7. The percentage of oxyproline is: gelatin, 14.7; zein, 0.8; and casein, 2.1. Damodaran (20) has proposed a scheme for determining the nitrogen distribution in protiens which includes the nitrogen present in the dicarboxylic amino acids. There is, however, nothing essentially new in the technique.

Since the sulfur-containing amino acids are being considered in another chapter, discussion of papers pertaining to them will be omitted from the present section.

Several papers relating to the racemization of amino acids have

appeared during the past year. Du Vigneaud & Sealock (21) showed that an aqueous solution of acetyltryptophane is racemized by acetic anhydride at 35°–40°. The acetyl *dl*-tryptophane could be resolved by means of *d*-*a*-phenylethylamine. The work on racemization was extended by du Vigneaud & Meyer (22) to include glutamic acid, phenylalanine, methionine, tyrosine, and arginine, and their formyl derivatives. Proline, however, is not racemized under these conditions. Du Vigneaud & Meyer (23) postulate that the mechanism followed in the process of racemization is the transitory formation of the azlactone of the acetylated amino acid.⁸ Racemization results whenever an azlactone of an acetylated amino acid which contains a hydrogen atom on the α-carbon atom is formed. This may be due to the migration of the hydrogen atom as represented by the shift from (II) to (III) or to the carbon of the acyl grouping as indicated by the conversion of (II) to (IV).



The hypothesis also accounts for the lack of racemization of proline since a true azlactone of an amino acid with a secondary amino group cannot be formed. The work of Csonka & Nicolet (24) points also to the formation of an azlactone during the process of racemization.

⁸ In this connection see Bergmann and Zervas, *Biochem. Z.*, 203, 280 (1928).

In 1931 Levene, Steiger & Marker (25) demonstrated that migration, under the influence of alkali, of the hydrogen atoms attached to asymmetric carbon atoms takes place only on the central amino acids of a polypeptide chain, the terminal amino acids which carry the free amino or the carboxyl group remaining wholly unaltered. For the racemization a minimum concentration of alkali was required to produce a significant change. Levene & Steiger (26) have continued this work. They show that the nature of the adjacent or all other amino acid units of the molecule affects somewhat the degree of racemization by alkali. Thus glycyl-*d*-leucyl-*d*-leucyl-*d*-leucine lost 23 per cent of its optical activity, while *d*-leucyl-*d*-leucyl-glycyl-glycine lost 19 per cent of its optical activity, after eight days when treated with 10 mols of 1.0 N NaOH at 25°.

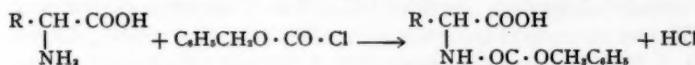
It is well known that in the hydrolysis of proteins either by enzymes or by hydrogen or hydroxyl ions certain amino acids are split off much easier than others. That the rate of hydrolysis of peptides in 0.5 N sodium hydroxide solution at 25° is dependent upon the chemical makeup has been shown in a series of studies by Levene and his co-workers (27). They obtained the following hydrolysis constants for a series of peptides: glycyl-glycine, 152×10^{-8} ; alanyl-glycine, 32×10^{-8} ; leucyl-glycine, 7.3×10^{-8} ; valyl-glycine, 0; glycyl-alanine, 37×10^{-8} ; glycyl-leucine, 9.3×10^{-8} ; glycyl-valine, 7.5×10^{-8} ; glycyl- α -aminoisobutyric acid, 0; alanyl-alanine, 6.5×10^{-8} ; leucyl-leucine, 0. A similar dependence on chemical makeup was shown by a series of diketopiperazines. The following are arranged in order of increasing ring stability: glycyl-glycine anhydride, alanyl-glycine anhydride, leucyl-glycine anhydride, valyl-glycine anhydride, and α -aminoisobutyryl-glycine anhydride.

Melting-point is a criterion most frequently used by chemists to identify organic substances. The method is less reliable when, on heating, decomposition occurs. This is one of the difficulties encountered with amino acids. Dunn & Brophy (28) have devised a method whereby the temperature at which a standard decomposition state of the amino acid, as judged by the shade of yellow or brown color produced on heating very rapidly, is determined with the aid of a thermocouple. The results appear to be reproducible within the limits of several degrees. The decomposition points found by Dunn & Brophy are: glycine, 289°–292°; *d*-glutamic acid, 247°–249°; *dl*-glutamic acid, 225°–227°; *L*-tyrosine, 342°–344°; *dl*-alanine, 297°; *dl*-phenylalanine, 318°–320°; *dl*-aspartic acid, 278°–280°; 3,5-diiodo-

l-tyrosine, 239°–241°; *l*-leucine, 337°; *dl*-leucine, 332°; *dl*-isoleucine, 292°; *dl*-norleucine, 327°; *dl*-valine, 292°; glycyl-glycine, 262°–264°; glycyl-glycyl-glycine, 262°–265°.

A number of analyses of proteins have been published. Such work when carried out with care and with the aid of precision methods is extremely valuable. Calvery (29) and Vickery & Shore (30) have reported analyses of hen's egg albumin. The percentages of amino acids found by the latter are: histidine, 1.4; arginine, 5.4; lysine, 5.0. The data agree with the assumption that the molecule of egg albumin whose molecular weight can be taken as 34,000 contains 3 histidyl radicals, 11 arginyl radicals, and 12 lysyl radicals. Stewart & Rimington (31) report the percentage of basic amino acids in wool as: histidine, 0.6; arginine, 6; and lysine, 2.2. Kondo & Ito (32) have published extensive analyses of the globulins of polished rice. Jukes & Kay (33) report analyses of livetin and vitellin. The basic nitrogen distribution in percentage of livetin is: arginine N, 10.3; histidine N, 2.1; lysine N, 5.9. Similar analyses for vitellin show: arginine N, 13.5; histidine N, 2.1; lysine N, 6.4. Livetin contains 2.3 per cent and vitellin 1.1 per cent of cystine. Livetin appears to be closely related to, if not identical with, the serum globulin of the common fowl (34, 35).

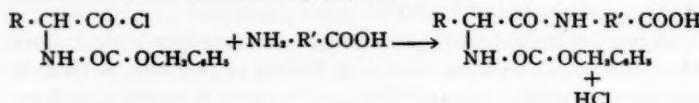
A new general method of far-reaching application for the synthesis of peptides has been announced by Bergmann & Zervas (36).⁴ They employ the chloride of benzylester carbonic acid (carbobenzoxy chloride) to mask the free amino group or groups of the acyl amino acid:



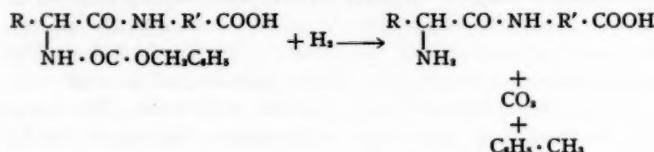
By converting the product so formed into either the acid chloride or

⁴ Bertho and his collaborators [*Ann.*, **485**, 127 (1931), and **498**, 50 (1932)] have also announced a method for the synthesis of polypeptides and amino-acid sugars. It involves catalytic reduction of the azide of the peptide. The peptide is prepared from the halogenated amino acid. One of the carbon atoms of the final product is, however, inactive. Abderhalden & Schwab [*Z. physiol. Chem.*, **212**, 61 (1932)] have prepared a number of peptides containing an amino acid and a diketopiperazine ring as represented by the compound *l*-leucyl-(glycyl-*l*-leucine anhydride). Bergmann, du Vigneaud & Zervas [*Ber.*, **62**, 1909 (1929)] have already indicated that diketopiperazines with amino-acid substituents on the nitrogen would not be stable in the free state. They believe that synthetic compounds of the type described by Abderhalden & Schwab should be formulated in some other way.

the azide, it can combine with the amino group of the second amino acid forming the carbobenzoxy peptide:



By shaking this solution for a few minutes with hydrogen gas and palladium at ordinary temperatures, the carbobenzoxy group is split off to carbon dioxide and toluol. The resulting optically active peptide is obtained in a quantitative yield and in pure crystalline form:



By the use of this method Bergmann and his collaborators have synthesized such complex peptides as aspartyl-tyrosine (36), glycyl- and alanyl-glucosamine (37), glycyl-proline (38), lysyl-glutamic acid, and lysyl-histidine (39), the synthesis of which could not be carried out by the older methods. The synthesis of such compounds permits the possibility among other things of a study of the correlation between chemical makeup and splitting by enzymes (40).

Greenstein (41) has reported further work on the dissociation constants of synthetic peptides (42). The dissociation constants at 25° are: (a) glycyl-tyrosine: pG'₁, 2.98; pG'₂, 8.40; pG'₃, 10.40; pI, 5.69; (b) tyrosyl-tyrosine: pG'₁, 3.52; pG'₂, 7.68; pG'₃, 9.80; pG'₄, 10.26; pI, 5.60. Hitchcock's values for tyrosine are: pG'₁, 2.20; pG'₂, 9.11; pG'₃, 10.07; pI, 5.66. The data point to the fact that the dissociation of the hydroxyphenyl group depends upon its relative position in the molecule with reference to the other free groups, being increased slightly by proximity to an amino group and decreased slightly by the nearness of a carboxyl group. The free amino and carboxyl groups in the tyrosine peptides are weaker as compared with similar groups in the amino acid. Further work on this subject will be published in the near future from the writer's laboratory. Greenstein (43) reports the dissociation constant of α-β-diaminopropionic acid as follows: pG'₁, 1.33; pG'₂, 6.80; pG'₃, 9.60; pI, 8.20. When these data are compared with the values reported for ornithine by

Schmidt, Kirk & Schmidt (44) and for lysine by Schmidt, Kirk & Appleman (45), it is seen that the molecule shows a progressively acid character as the distance between amino groups diminishes. When the logarithms of the dissociation constants are plotted against the reciprocal of the number of carbon atoms separating the charged groups, a straight line is obtained. A similar relationship was previously shown by Schmidt, Appleman & Kirk (46). In this connection, the experiments of Dunn & Schmidt (47), showing that the time required for an amino acid to react quantitatively with nitrous acid varies directly as the distance of the amino group from the carboxyl group, are of interest. Richardson (48) has determined the dissociation constants of *dl*- β -hydroxy-glutamic acid at 25° as follows: pG'_1 , 2.10; pG'_2 , 4.17; pG'_3 , 9.20. The dissociation constants for hydroxy-aspartic acid and the hydroxy-asparagines were published in 1930 by Chibnall & Cannan (49).

Czarnetzky & Schmidt (50) have reported the apparent dissociation constants at 25° for several amino acids. The data are: (a) Oxyvaline: pK'_a , 9.71; pK'_b , 11.39; pI , 6.15; (b) α -amino-*n*-butyric acid: pK'_a , 9.60; pK'_b , 11.45; pI , 6.07; (c) oxy- α -aminobutyric acid: pK'_a , 9.62; pK'_b , 11.29; pI , 6.15. Derrick factors were calculated as follows: glycine, 1; alanine, 1.08; α -amino-*n*-butyric acid, 0.90; and α -amino-*n*-valeric acid, 1.07. They have also published values for $(\Delta F^\circ)'$, $(\Delta H)'$, and $(\Delta S)'$. Similar data for the other amino acids have been published by Miyamoto & Schmidt (51). Huffman & Borsook (52) have determined the heat capacities, entropies, and free energies of *d*-alanine, *l*-asparagine, *l*-asparagine hydrate, *l*-aspartic acid, and *d*-glutamic acid. Lack of space prevents inclusion of the thermodynamic data relating to the amino acids.⁵

Winnek & Schmidt (53) find the following dissociation constants at 25° for dibromtyrosine: pK'_{a_1} , 6.22; pK'_{a_2} , 7.39; pK'_{b_1} , 11.83; pI , 4.18.

Further evidence tending to show that proteins form complex ions with metallic elements has appeared (54). The earlier literature on this subject has been reviewed by Smythe & Schmidt (55). Borsook & Thimann (56) have studied with the aid of copper-electrode potentials and by measurement of the absorption in the visible spectrum the equilibrium which exists in solution between cupric ions and glycine and alanine and the influence of variations of pH thereon.

⁵ Work on the determination of heat of solution, specific heat, and heat of dilution of the amino acids is being carried out in our laboratory by Mr. Zittle.

They conclude that each amino acid forms at least four types of complexes with cupric ions, these being dependent upon the pH of the solution. It is not quite clear to the writer why four compounds rather than a series of the type which has been described for iron by Smythe & Schmidt (55) should be formed. Borsook & Thimann were apparently unaware of these studies. Ettisch & Schulz (57) have recently shown by means of copper-electrode measurements the dependence of the amount of copper bound by certain proteins upon the pH of the solutions. The reader should also note the work of Failey (67) on thallium, reference to which is made elsewhere in this article.

Miyamoto & Schmidt (58) have again taken up the question as to the seat of the formation of the complex ions of the alkaline-earth elements and casein. They carried out transport experiments on solutions of sodium caseinate, sodium dephosphorized caseinate, calcium caseinate, calcium dephosphorized caseinate, and the sodium, barium, and calcium salts respectively of glutamic acid and of aspartic acid. Their data show that of the foregoing solutions only the calcium salts of casein and of dephosphorized casein yield complex ions, the latter about 10 per cent less than the former. It is concluded that the phosphoric acid in casein is responsible for this difference. They advance the hypothesis that solutions of casein in the alkaline-earth elements yield complex ions due to step dissociation. They also determined the conductivities of various salts of casein, dephosphorized casein, aspartic acid, and glutamic acid as well as of free aspartic acid, glutamic acid, and glycine. By plotting $(C \wedge)^{\frac{1}{2}}$ against $\frac{1}{\wedge}$, a straight line was obtained. A similar relationship has been found by Hayashi (59). The conductivity data were also plotted with the aid of Onsager's (60) modification of the Debye-Hückel theory for the conductivity of strong electrolytes and the same type of deviation from a straight line as shown by strong electrolytes was found, indicating a similarity in behavior. They also attempted to apply Ostwald's dilution law to solutions of the above-mentioned free amino acids. The results show that these amino acids differ from acetic acid in that they obey the law only in extremely high dilutions, whereas acetic acid follows the law in comparatively low dilutions. A satisfactory agreement between the mobilities of the various protein and amino-acid anions studied, as determined by conductivity and transference experiments, was obtained. The following are the average anionic mobilities: sodium caseinate, 44.9; sodium dephos-

phorized caseinate, 37.1; monosodium aspartate, 27.7; barium diaspartate, 27.6; calcium diaspartate, 27.7; monosodium glutamate, 25.1; barium diglutamate, 25.1; calcium diglutamate, 25.1; aspartic acid, 27.8; and glutamic acid, 25.1. From conductivity measurements carried out on aspartic acid and on glutamic acid solutions at 0° and 25°, Miyamoto & Schmidt (61) have determined the temperature coefficient for the aspartate ion as 0.0195, and for the glutamate ion as 0.0198. König & Pauli (62) measured the mobility of the ions which are formed when different amounts of sodium hydroxide or of hydrochloric acid are added respectively to serum albumin, pseudoglobulin, and ovalbumin. The mobility increases with the amounts of acid or base added or, in other words, with the charge carried by the protein ion. The order of magnitude of the mobilities is less than that reported from this laboratory for casein (63) and for fibrin (64), proteins which are insoluble in water at their isoelectric points, and more nearly that of aspartate and glutamate ions (58). The experimental error in König & Pauli's work appears to be in many instances considerable.

According to Abramson (65), in solutions of the same ionic strength, the electric mobilities of the same protein at different hydrogen-ion activities should be proportional to the number of hydrogen or hydroxyl ions bound, a rule which was found to hold for egg albumin, serum albumin, casein, gelatin, and deaminized gelatin.

A number of individuals have reported further work on the combination of proteins, peptids, and amino acids with acids and with bases. Hitchcock (66) carried out electromotive-force measurements without liquid-junction potentials of the type Ag, AgCl, HCl + protein, H₂, at 30°. His data for the combining capacities for hydrogen ion are: edestin, 13.4×10^{-4} ; gelatin, 9.6×10^{-4} ; and casein, 8.0×10^{-4} equivalents of combined H⁺ per gram of protein. He interprets his data on the assumption that none of the protein combines with the chloride ion, that each protein has a definite maximal combining capacity for hydrogen ion and that the mean activity coefficient of the ions of hydrochloric acid is decreased by the presence of the protein in such a way that its logarithm is a linear function of the protein concentration, a principle which has been proposed by Failey (67). Robinson, Gortner & Palmer (68) have again taken up the question of the mode of combination of proteins with alkali, especially at high alkalinites. Although admitting that, while stoichiometrical factors are concerned in the combination of protein with

acid and with base, they consider that other factors, possibly an adsorption type of reaction, enter, especially in the region of high acidity and of high alkalinity. It is doubtful as to whether this question can ever be satisfactorily settled by the technique which these workers have employed, viz. potentiometric titration with the aid of the hydrogen electrode. In regions of high acidity and alkalinity the method is not very accurate. There is the uncertainty of the contact potential. In regions of high acidity and alkalinity the activity of the hydrogen ion is probably not equal to its concentration. Moreover, at best, the method measures an equilibrium rather than the total combining capacity of proteins for acid or alkali. To overcome this difficulty, Chapman, Greenberg & Schmidt (69) and Rawlins & Schmidt (70) measured the combining capacity of proteins for acids and for bases by determining the amount of dye combined on precipitation. However, even this method does not determine all of the acid or the base with which a protein can combine. Experiments which are being carried out in this laboratory by Czarnetzky & Schmidt (71) show that when dry protein is treated with dry hydrogen chloride, the amount of this gas with which the protein combines is greater than can be accounted for on the basis of the free basic groups of the protein molecule. The indications are that other groups besides those hitherto described combine with this gas. We may cite as an example the fact that under ordinary conditions histidine combines with two mols of hydrochloric acid to yield the dichloride, yet when treated in the dry state with hydrogen chloride, it can combine with three mols of this acid. Histidine probably exists in several tautomeric forms which are in equilibrium with each other. The third molecule of hydrogen chloride probably attaches itself to the double bond. Robinson, Gortner & Palmer point out the influence of the presence of neutral salts upon the pH of proteins dissolved in alkali. This is itself a criticism of the method of determining combining capacities and is a factor which has probably unconsciously entered into a good many estimations of pH.

Felix & Reindl (72) have employed the method of methylation to determine the free groups present in gliadin. They use the methoxy content of the ester as a measure of the free carboxyl groups and the chlor content of the ester as a measure of the basic groups. In gliadin they find 19.5×10^{-4} equivalents for the acid groups and 12.3×10^{-4} equivalents for the basic groups per gram of protein. In the thymus histone of the calf, Felix & Rauch (73) find 7.5 free carboxyl groups

and 20 equivalents of basic groups per 100 atoms of nitrogen. Dirr & Felix (74) report that the number of benzoyl groups taken up by clupein is measured by the content of arginine (where it is attached to the free guanidine groups) and the content of monoamino acids (where it is probably attached to the free hydroxyl groups of serine). According to Felix & Kahlert (75), globin contains 12.8 acid and 13.2 basic groups per 100 atoms of nitrogen.

Considerable work indicating that certain of the enzymes and other products of biological importance are protein in nature has been done. Thus Northrup has brought forth considerable evidence that pepsin (76) and trypsin (77) are proteins. On mild hydrolysis of pepsin the decrease in activity is proportional to the decrease of the protein nitrogen of the solution. Loss of tryptic activity corresponds to the decrease in native protein when the enzyme is denatured by heat, digested by pepsin, or hydrolyzed in dilute alkali. The molecular weight is about 34,000 and its isoelectric point lies between pH 7 and 8. According to Sumner, Kirk & Howell (78), crystalline urease is rapidly inactivated by pepsin and by papain-H₂S, and this inactivation coincides with proteolysis. The investigations which have been carried out by Freudenberg and his co-workers (79) and others point to the protein nature of insulin. Its activity is destroyed by proteolytic enzymes, sodium amalgam, aromatic aldehydes, hydrogen peroxide, iodine (80), irradiation, acids and alkalies, by methylation, and by treatment with cysteine or glutathione (81). It contains free amino and free carboxyl groups. Some of the nitrogen may be split off in the form of ammonia without loss of activity. Treatment with nitrous acid to remove free amino nitrogen is likewise without effect on activity. When loss of activity occurs, it is probably due to oxidation by the nitrous acid. Its molecular weight is either 18,000 (82) or a multiple thereof (83). It contains 14.5 per cent of nitrogen, of which 1 per cent is in the form of amino groups. Analysis by Jensen & Wintersteiner (84) shows the following percentage content of amino acids (uncorrected for water): tyrosine 12, cystine 12, glutamic acid 21, leucine 30, arginine 3, histidine 8, and lysine 2.

A number of papers dealing with hemoglobin have appeared. Drabkin & Austin (85) have determined the spectrophotometric constants for the common hemoglobin derivatives in human, dog, and rabbit blood. Conant & Pappenheimer (86) have redetermined the oxidation potential in mixtures of hemoglobin and methemoglobin. At the midpoint of their curve the concentrations of hemoglobin and

methemoglobin were 3.5 milli equivalents per liter, $\mu = 0.3$, pH 7.0, and $T = 24^\circ$; the value of E_0 was found to be 152 ± 5 millivolts. Tadokoro, Abe & Yoshimura (87) believe that crystalline horse hemoglobin occurs in three isomeric forms: (a) one form soluble in cold water, (b) one form soluble with difficulty, and (c) one form insoluble at 30° . They can be further differentiated by their isoelectric points and the composition of the globin molecules. Green (88) has studied the solubility of horse carboxy-hemoglobin in various chloride and sulfate solutions. At pH 6.6 and 25° the solubility can be expressed by the equation

$$\log S = 1.30 + 1.6 \sqrt{C} - k_0 C$$

where k_0 is an empirical constant varying with the electrolyte. The activity-coefficient of hemoglobin in these salt solutions can be approximately described in terms of a simplified Debye-Hückel equation

$$-\log \gamma = \log S - \log S_0 = \frac{0.5 Z_1 Z_2 \sqrt{\mu}}{1 + A} - K_s$$

where $\log S_0 = 1.20$, $Z_1 Z_2$ is 4, A and K_s are empirical constants.

A good deal of our knowledge relating to the isolation, identification, and quantitative estimation of serum albumin and serum globulin, as well as their state in unaltered serum, is based on the fractional precipitation of these substances. Now it is to be expected that such precipitations are not sharply defined. The converse procedure is to study the solubility of the serum proteins in different concentrations of salt solutions. For this purpose Butler & Montgomery (89) have employed potassium phosphate solutions since it is possible by their use to vary independently pH, salt concentration, serum concentration, and temperature and thus express the solubility of the saturating body as a function of any one of these variables. Florkin (90) and Green (91) have also used phosphate solutions in protein-solubility studies. Butler & Montgomery find no evidence of the existence of two pseudoglobulins in horse serum such as Howe (92) reported for calf serum. On the assumption that horse plasma contains protein complexes from which fibrinogen, euglobulin, pseudoglobulin, and albumin can be salted out, the data when plotted are similar to a hypothetical solubility-curve constructed on the assumption that the individual protein complexes separate out similarly to pure proteins in concentrated salt solutions. Their results also point to a difference in the behavior of the globulin of human plasma and the pseudo-

globulin of horse plasma. In many instances there is a decided difference between the analytically obtained values for the percentages of globulin and albumin in serum depending on the mode of precipitation, showing that the definition of these fractions based on salting-out procedures is still in large part arbitrary.

Cohn (93) has summarized in an excellent way the factors which are concerned in the solubility of proteins and amino acids. Besides temperature these are (*a*) nature of the molecule, which includes such factors as molecular weight, free groups, and especially the length of the hydrocarbon chain, (*b*) dipole moment, and (*c*) dielectric constant. Amino acids markedly increase the dielectric constant of water. The effect of increasing the size of the molecule has no marked influence on the dielectric constant. On the other hand, length of the hydrocarbon chain has a marked influence on solubility. Thus glycine is more soluble in water than α -alanine. In alcohol-water mixtures the amino acids with the longer chains are more soluble. Chemical makeup, probably on account of the presence of specific groups, possesses a very great influence on solubility. Thus proline is quite soluble in water and in alcohol. The prolamines are more soluble in alcohol-water mixtures than in water or in pure alcohol. The dipole-moment influence is seen on comparing the solubility of α -alanine and β -alanine. The latter, with the greater dipole moment, is the more soluble in water. The effect per mol of β -alanine on the dielectric constant of water is nearly twice that of α -alanine. The solubility in 90 per cent alcohol increases with increasing dipole moment. The influence on solubility of inorganic salts varies with the nature of the salt and the amino acid. In general, amino acids whose solubilities are decreased most by alcohol are more soluble in salt solutions than in water. The solubility relationships of proteins resemble in many ways those of the amino acids. The solubility is determined by the chemical makeup and size of the protein molecule, and the nature and concentration of the neutral salts. When the concentration of neutral salt is increased sufficiently, salting-out occurs. The relations can be expressed by the equation

$$\log S = \beta - K' \mu$$

where S = solubility, μ = the ionic strength, β = an intercept constant, and K' = salting-out constant. The relations have been experimentally verified by Florkin's (90) studies on fibrin and Green's (91) work on hemoglobin.

A good deal of information as to the state of the protein molecule in its isoelectric condition in urea, in salt solutions, when denatured, and when coagulated, has been gained from the determination of the molecular weight. Comparison of such values with the molecular weight as calculated from the content of a metallic element such as iron (hemoglobin), copper (hemocyanin), or an amino acid which is present in minimum amounts yields evidence as to the state of aggregation of the substance in question. Burk (95) has recently carried out an extensive investigation of the molecular weight of serum albumin. This substance possesses the advantage of solubility in water, salt solutions, glycerol, and urea (in which it is, however, denatured); though it is coagulated by heat, the coagulated protein is soluble in urea solution. Burk found a mean molecular weight of 74,600 for serum albumin which is in good agreement with the value of 72,000 reported by Adair & Robinson (96) and 78,000, the value which can be calculated from the tryptophane content. Essentially the same molecular weights were obtained irrespective of the nature of the solvent employed or whether the protein was denatured or coagulated. It was also found that in moderately concentrated salt solution the osmotic pressure of serum albumin does not conform to the ideal law but increases more than proportional to increases in protein concentration. When corrected for this deviation a value for the molecular weight as reported above is obtained. Burk, moreover, reports that repeated recrystallization of serum albumin does not lead to a change in molecular weight, which points to the stability of this protein, findings which are at variance from those reported by Svedberg & Sjögren (97). The constancy of the molecular weight after repeated purification may be indicative of constant composition. It cannot be taken as absolute proof, however, since the molecular weight is after all merely a statistical average for all of the molecules present in solution.

Svedberg & Eriksson⁶ (98) have determined by means of the ultracentrifuge that the molecular weight of *c*-phycocyan is 208,000 and *r*-phycoerythrin is 196,000. They, moreover, report that the molecular weight of *r*-phycocyan varies with the pH of the solution. In the region of pH 2.5 to 5.0 the molecular weight is 209,000, while in the region less acid than pH 1.5 and less alkaline than pH 8.0 the molecular weight is about half of this value. In the transitory regions

⁶ A more complete bibliography to the work of Svedberg and his collaborators is given by H. B. Vickery, *Yale J. Biol. Med.*, **4**, 595 (1932).

of acidity or alkalinity, mixtures of these two molecular weights were obtained.

It is strongly suspected by the writer that sufficient attention has not been paid to the idea that extensive aggregation or micellation occurs in protein solutions.⁷ This does not mean the acceptance of Svedberg's (99) idea that protein molecules are built up of units whose molecular weight is about 34,500. It is, of course, true that in a number of instances the molecular weights are multiples of this number. This must not, however, be taken too seriously since many large numbers are approximately multiples of 34,500. Moreover, a sufficiently large number of proteins has not yet been investigated to make this rule a general one. The amount of aggregation in a protein solution is possibly dependent on the pH of the solution, concentration, the presence of both electrolytes and non-electrolytes, and other proteins. A criticism which may be directed against the idea of micellation is that it does not account for the forces involved nor the mechanism whereby micelles are formed. The presence of micelles in a solution is not to be regarded as evidence for a static system but rather a dynamic one involving equilibria of which statistical averages are merely determined.

Whether the protein which is obtained after repeated purification is the same as that which exists in the native state is also an open question. Svedberg (100) believes that in certain instances they may be the same but in other instances such as lactalbumin and ovalbumin their makeup is markedly influenced by the method of preparation. It is not possible in the present state of our knowledge to pass final judgment on this issue. It is very important that as many criteria as possible be applied to proteins to test their homogeneity and chemical stability. The writer believes that much relating to this problem may be learned by studies carried out on solutions of polypeptides, many of which are fairly resistant to the hydrolytic influence of acidity and alkalinity and to the study of which some of the physico-chemical methods which are not applicable to proteins can be applied.

A good deal of data has been brought forward in recent years,

⁷ Hoskins, Randall, and Schmidt [*J. Biol. Chem.*, **88**, 215 (1930)] have shown that the undissociated part of aspartic acid and of glutamic acid exists to a considerable extent as neutral aggregates, while the ionized part of the mono-sodium salts of these amino acids in solution probably exists to a slight extent as ionic aggregates or micelles. See also M. Fränkel, *Biochem. Z.*, **217**, 378 (1930); and W. C. M. Lewis, *Chem. Rev.*, **8**, 81 (1931).

the substance of which is that a number of the well-known proteins are not chemical entities (101). Evidence in this field has led Sörensen (102) to propose that many if not all of the proteins can be regarded as reversibly dissociable systems of components. The components differ in minor respects. The protein in its native soluble state is an aggregate of components held together by secondary valence forces. It is undoubtedly true that the composition of many of our proteins varies with the mode of preparation. Others such as egg albumin and serum albumin [as shown by the work of Burk (95)] appear to be quite stable. While there is much to be said in favor of Sörensen's theory, the data at hand are still too few to warrant its general acceptance. Among other questions which must be elucidated is the nature of the valence forces which hold the components together, the point of attachment of the units, and the conditions which favor the combination.

Although much information concerning the subject of denaturation has been brought out in recent years, the mechanism is by no means clear. Denaturation is chiefly characterized by insolubility at the isoelectric point in a solution of a native protein. It may or may not be accompanied by chemical changes (103). According to Anson & Mirsky (104), the condition is a reversible one. Denaturation leads to increase of viscosity (105). Adsorption by aluminum hydroxide or gum mastic may lead to denaturation (106). Burk & Greenberg (107) found that denatured hemoglobin in urea solution possesses half the molecular weight of hemoglobin when dissolved in water. The experiments of Burk (95) indicate no effect of denaturation on molecular weight. However, in his experiments, the possibility of reversion having taken place is perhaps not excluded. According to Loughlin & Lewis (108) denaturation consists in a change from the molecular to a micellar state. At the isoelectric point denaturation is accompanied by an increase in the volume of the protein unit. If one accepts all of this evidence at face value, then it is evident that, from the molecular standpoint, the phenomenon is not a uniform one. Evidently, further data especially by the use of other criteria must be brought forth before the problem can be considered a closed one (109).

Anslow & Foster (109) have studied the absorption of light-energy of different frequencies by amino acids in order to discover the particular groups whose linkage is weakened by light-absorption. Their data for the energy of dissociation (volts) are: alanine, 6.14; cysteine, 5.77; aspartic acid, 5.66; glutamic acid, 5.55; cystine in

HCl, 5.49; cystine in H₂O, 5.62. It is shown that symmetrically formed molecules absorb light-energy selectively, weakening the linkage between the α- and β-carbon groups, as in the case of aspartic and glutamic acids, and between the -S-S- groups in cystine. In the case of cystine in HCl solution, the disruption leads to the formation of cysteine chloride and cysteine and, in water, cysteine hydroxide and cysteine. In this connection, it is of interest to recall the experiments of Stenström & Lohmann (110), who showed that cystine solutions in H₂SO₄ were unaffected by exposure to X-rays while the phenol group of tyrosine is affected. Bernal (111) and Hengstenberg & Lenel (112) have supplied data for the characterization of the crystal structure of a number of the amino acids and peptides.

A review of the work of Brill (113), Ott (114), and Astbury & Woods (115) and others on X-ray interference diagrams of silk and of wool is given by Vickery (116) and by Rimington (117). Normal wool fiber exhibits a spacing of 5.15 Å along the fiber axis with true side spacings of 9.8 Å and 27 Å. During the process of stretching in the moist condition, a new periodicity becomes apparent which has a characteristic period of 3.4 Å with a side spacing of 9.8 Å. Two atoms which in normal wool are at a distance of 5.15 Å are at a distance of 10.2 Å, which corresponds to a stretching of about 100 per cent. This means that in the unstretched condition the molecule may be contracted by the formation of loops which need not necessarily be closed and which lie along the axis of the molecule. On stretching, these loops would provide the means for the molecular elasticity. Silk fiber shows a periodicity of 3.5 Å. The periodicity is about the same as that of stretched wool and points to the fact that silk fiber does not exhibit the same elasticity as wool. In fibers such as silk and wool, physical forces have led to an orderly arrangement of the structural units; the orderly arrangement may, however, not exist in water-soluble proteins, since there is no orienting field except water and the air-water interface. Attempts have been made to bring out evidence favorable to the idea that the protein complex consists of peptide chains characteristic of the protein in question which are grouped together and held by covalence forces in micelles of 2.2 μμ radius and of 34,500 molecular weight (the Svedberg unit). These units correspond somewhat to the crystallites of cellulose but possess a weaker power of association, this being dependent upon the pH and other characteristics of the micelle. Further evidence in support of this is the observation by Gorter & Grendel (118) that when proteins

are permitted to spread upon the surface of weak hydrochloric acid the same area is occupied by all irrespective of particle mass. On the basis of the Svedberg unit they obtain a value of 22.5 Å for the radius of the unit particle, which is the same as that found by Svedberg. In spreading, the forces which bring about surface orientation are sufficient to overcome intermicellar cohesion and produce micellar units whose size is that of the Svedberg unit. The interesting thing about the various hypotheses, viz. of Svedberg, Astbury & Woods, Sörensen, and Gorter & Grendel, is reversibility. This reminds one of denaturation. It is possible that among other changes which are involved in this process, orientation toward water molecules may be one. The molecular weight may remain the same, or, in certain instances, it may involve a decrease by demicellulation or an increase by micellation.

A review of his interesting work on clupein has been published by Felix (119). It appears that, although clupein is a comparatively simple substance, it is not a homogeneous one. Clupein A₁ contains 4 mols of arginine, and 1 each of serine and alanine. Clupein A₂ contains 4 mols of arginine and 1 each of proline and aminovaleric acid. Clupein B contains a mol each of clupein A₁ and A₂ minus a mol of water, while clupein C contains 2 mols each of clupein B minus a mol of water. Sturine probably contains 11 mols of arginine, 2 mols of histidine, 3 mols of lysine, 3 mols of alanine, and 1 mol of leucine.

The work of Craig & Schmidt (94) on the optical refractivity of solutions of amino acids and proteins mentioned in last year's review has been published during the past year.

In a recent theoretical article, Scatchard & Kirkwood (120) have taken up the problem of the electrostatic forces involved in the interaction of electrolytes and zwitterions. The authors find that the effect of the distance between the charges on the same molecule varies with the concentration of the electrolyte and in such a way that the limiting Debye-Hückel law depends neither on the distance between the charges nor on the shape of the molecule but only on the total charge. The action of a zwitterion in dilute solution is proportional to the concentration and not to its square root. Any polyvalent ion will behave approximately like an ion with spherical symmetry whose diameter depends both on molecular size and the distance between the ions.

Greenberg & Greenberg (121) have shown that ultrafiltration of diffusible electrolytes from systems containing electrically charged colloids such as the alkali caseinates partakes of the nature of a Donnan membrane distribution.

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THE CHEMISTRY AND METABOLISM OF THE COMPOUNDS OF SULFUR*

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ERGOTHIONEINE

A method for the determination of ergothioneine in protein-free filtrates from the corpuscles of unlaked blood has been suggested by Salt (1). Wide variations (3.05–12.00 mg. per 100 cc. of corpuscles) were observed in normal human blood. The previously reported high values in pig blood were confirmed (15.6–24.2 mg. per 100 cc. of corpuscles). Behre (2) has criticized the use of corpuscular filtrates and believes that Salt's method, less simple than the procedure in which filtrates from whole blood are used, does not yield figures of sufficiently greater accuracy to justify the use of the cumbersome procedure involved. A more rapid and more satisfactory method than those previously employed for the isolation of ergothioneine from pig blood (3) has yielded about 85 per cent of the amount present as determined quantitatively; the method can be applied to bloods containing as low as 20 mg. per liter.

METHIONINE

The occurrence of *L*-methionine as a primary product of hydrolysis of the protein molecule has been established (4) by its isolation from the products of the tryptic digestion of casein (1–2 gm. per kilo). Methionine has been isolated (5) in small amounts from the products of acid hydrolysis of ox horn. Pirie (6) has utilized the solubility of methionine in butyl alcohol to effect a partial separation of methionine from certain other amino acids, the presence of which make the isolation of methionine more difficult, and by this and other modifications has greatly increased the yield of methionine which can be obtained from casein by acid hydrolysis (1–1.4 per cent). The sulfur content of the methionine actually isolated corresponded to approximately 50 per cent of the total sulfur of the casein.

The need of an accurate method for the quantitative determination of methionine in proteins has been met in part by Baernstein (7),

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who has reported studies of the sulfur distribution in proteins (8) with the use of this method for the determination of methionine. A technique for the determination of the volatile iodide liberated when proteins are treated with hydriodic acid was developed. Since no methyloxyl or methylimide groups are known to occur in the protein molecule, it was assumed that the methyl iodide obtained was derived solely from, and was a measure of, the methylthiol group of methionine. With eleven amino acids other than methionine, the results were all negative, while standardization of the method with pure synthetic methionine gave satisfactory recovery (97.6 ± 1.4 per cent). Despite the possible error involved in the assumption that the methyl iodide liberated is a product of demethylation of methionine only, it is believed that the application of this method to the analysis of proteins has given results of significance. The methionine content of thirty-two purified proteins of known origin (7) ranged from 0.54 (arachin) to 4.47 per cent (ovalbumin). In general, proteins from animal sources showed a higher methionine content than did the proteins of the plant world.

Methionine, on heating with strong sulfuric acid (12–18 N), was found to yield a product (9) which gave a positive reaction in the Folin-Marenzi method for cystine determination, a positive cyanide-nitroprusside test, but no reaction in the Sullivan test for cystine. No similar product was formed after heating with hydrochloric acid. From the products of the reaction with sulfuric acid, a new sulfur-containing amino acid was isolated—homocystine, so called because it appeared to be the next higher symmetrical homologue of cystine [bis-(γ -amino- γ -carboxypropyl) disulfide]. The possibility of the formation of homocystine from methionine in acid hydrolysis of proteins or of the occurrence of the amino acid itself in the protein molecule must be borne in mind.

CYSTINE AND CYSTEINE

A new test for cysteine (or cystine when reduced by tin and hydrochloric acid), by which ten parts per million may be detected, has been suggested (10). If an aqueous solution of cysteine hydrochloride is shaken with a chloroform solution of *o*-benzoquinone, a red color appears in the chloroform layer. This test may be used to detect cysteine in the presence of cystine, glutathione, and many other compounds containing nitrogen and sulfur. Its specificity has been questioned (11), since, although the reaction may serve to

differentiate cysteine from the chief amino acids of biological importance, positive results may also be obtained with benzidine, aniline, many aliphatic amines,¹ and other substances.

In a study of the reaction of cysteine with iodine (12)—a reaction which forms the basis of the various iodometric methods proposed for the determination of cysteine (Okuda, etc.)—the iodine consumption in low concentrations of hydrochloric acid exceeded the theoretical amount required for the oxidation of the —SH groups to the —SS— group. The explanation appears to lie in the oxidation of the cystine formed, since iodine was shown to oxidize cystine in aqueous solution to cysteic acid quantitatively, the cysteic acid being isolated and identified by analysis and a study of its properties ($\text{RSSR} + 5\text{I}_2 + 6\text{H}_2\text{O} = 2\text{RSO}_3\text{H} + 10\text{HI}$). Andrews (13) has studied the oxidation of cystine by air in acid solutions. After ninety months in the presence of 6 N hydrochloric acid (20 per cent) at 38°, cysteic acid equivalent to 78 per cent of the original cystine was isolated and identified. No evidence of this formation of cysteic acid, apparently a reaction involving atmospheric oxidation, was obtained in solutions containing sulfuric acid.

One of the standard methods for cystine determination (Folin-Marenzi) has utilized sodium sulfite for the reduction of cystine to cysteine. A more detailed study of this reduction (14) has shown that no sulfate is formed, that only half of the cystine is converted to cysteine, and that the remainder appears as a new compound, S-cysteine-sulfonic acid [$\text{COOH} \cdot \text{CH}(\text{NH}_2) \cdot \text{CH}_2\text{SSO}_3\text{H}$].

Preisler and Preisler (15) have demonstrated that dithio acids (e.g., dithiohydroacrylic acid) may be acted upon by cupric salts (sulfate or perchlorate) with the formation of the cuprous salt of the thiol acid, the sulfonic acid, and the sulfuric acid—an interesting example of the interaction of two substances in higher states of oxidation to produce more reactive reducing substances and some of a more fully oxidized unreactive product. The importance of further study of this reaction in connection with the use of cupric or cuprous salts in the isolation of RSSR or RSH compounds (in the presence

¹ After this review was submitted for publication, it was shown by Baudisch and Dyer [J. Biol. Chem., 99, 485 (1933)] that piperidine and methylamine (and presumably other aliphatic primary amines) did not react positively in the *o*-benzoquinone test in the presence of 0.2 N hydrochloric acid. The reaction of cysteine, aniline, or benzidine was not influenced by the presence of hydrochloric acid.

of oxidizing agents) is obvious. A similar study of the reaction between mercuric salts (bromide) and dithio acids has been reported (16).

Clarke and Inouye (17) observed that the course of the alkali decomposition of S-substituted cysteines was in general similar to that of cystine and cysteine under similar conditions. The chief factor in the stability of compounds of this type toward alkali appeared to be the degree of unsaturation of the group attached to the sulfur atom. This difference in unsaturation may explain the greater reactivity of cystine in alkaline solution as compared to that of cysteine. Cysteic acid was similarly decomposed by alkaline reagents but much more slowly than cysteine.

From a study of the absorption spectra of amino acids and related compounds (18), it has been postulated that symmetrically formed molecules may absorb light energy selectively and weaken the linkage between α - and β -carbon groups, or, in the case of cystine, between the sulfurs of the disulfide linkage. With wave-lengths of approximately 2500 Å, cystine in hydrochloric-acid solution is believed to be split into cysteine chloride and cysteine or in aqueous solution to cysteine hydroxide and cysteine. The specific rotations of cystine, cysteine, and glutathione with mercury green light have been measured accurately within the pH range of from 2.1 to 9.0 (19).

Abderhalden and Geidel (20), by the use of oxidized glutathione as the initial cystine-containing peptide, have synthesized peptides of high molecular weight which contain cystine. The largest of these, di-(glycyl-*l*-leucyl-glycyl-*l*-leucyl)-glutathione (oxidized), contained thirteen molecules of amino acids with the high molecular weight of 1293. It displayed no colloidal properties and was readily soluble in water, despite the presence of relatively insoluble amino acids in the molecule. By the use of the new method of peptide synthesis of Bergmann and Zervas (21), it should be possible to prepare a wide variety of cystine-containing peptides, notably those in which the carboxyl groups of cystine are bound in the peptide linkage. The study of the chemical and biological properties of cystine in peptides of this type should prove of unusual interest.

Tompsett (22) has modified the Folin-Marenzi method for the determination of cystine in proteins by the substitution of saturated sodium bicarbonate for sodium carbonate as the alkali. The color is stated to be more stable and turbidity does not develop, so that the use of lithium sulfate to prevent clouding is unnecessary. None of

the methods proposed for cystine determination is entirely satisfactory. A new method adequate from the standpoints of specificity, accuracy, and simplicity is urgently needed.

SULFUR OF THE PROTEINS

In view of the importance of the cystine content of the diet in relation to the growth of wool and hair as a practical problem of animal husbandry, studies of the cystine content of the proteins of pasture grasses have been undertaken (23, 24). Analysis of fresh pasture grass (23) by the Sullivan and the Folin-Marenzi methods showed the presence of traces only of cystine (estimated at less than 0.1 per cent of the dry weight of the grass). A study of the proteins of cocksfoot grass (sulfur content, 0.75-1.0 per cent) likewise demonstrated the virtual absence of cystine (24) and led to the suggestion that the protein of cocksfoot grass must contain a non-basic sulfur-containing amino acid (e.g., methionine). Since grass plays a most important rôle in the diet of sheep in the grazing areas, the conclusion that "some precursor of cystine is present in grass, which can be converted to cystine in the animal body" (23), and thus contribute to the formation in the sheep of the cystine-rich epidermal tissue, wool, seems justified.

Hsieh (25) has reported the percentages of cystine obtained by isolation from hair in China. Notable is the extremely low value (1.5-2.5 per cent) for cow's hair. Other figures resemble those previously obtained. Analytical data of the cystine content of the glutelins of the more common cereals (26), as determined by a modification of Sullivan's method, ranged from 0.516 (α -glutelin of corn) to 1.440 per cent (α -glutelin of wheat).

The problem of the distribution of the sulfur in the protein molecule has been of interest since the early work of Schulze and of Osborne concerned with the loosely combined sulfur of the proteins. The recognition of methionine as a protein unit of widespread occurrence and the elaboration of a method for its quantitative determination (7) have resulted in renewed study of the problem. Baernstein (8) has determined the total sulfur, sulphydryl and disulfide sulfur (cystine), and methionine sulfur in a large number of proteins important nutritionally and of common occurrence. It is recognized that the values obtained for —SH and —SS— groups (chiefly cystine) might have included also the groups present in cysteine and thiolhistidine, but the evidence for the presence of these

amino acids is still far from convincing. In casein, a protein notable for a very low content of cystine and an average total sulfur content, the methionine sulfur was 84.2, 84.2, and 90.2 per cent of the total sulfur in three samples of different origin. In ovalbumin, also a protein known to contain little cystine, 61.4 per cent of the sulfur was present as methionine sulfur. In lactalbumin, a protein with a higher cystine content than any of the common proteins except the keratins, only 36.3 per cent of the total sulfur was accounted for as methionine sulfur. The recovery of total sulfur as methionine and sulphydryl and disulfide averaged 101.5 ± 6.6 per cent. Despite possible errors in analytical procedure, this study is an important advance in our knowledge of the nature of protein sulfur from the quantitative standpoint.

The cystine (and sulfur) content of the peptide insulin is still under investigation. Jensen and Wintersteiner (27) have been able to account for 88 per cent of the molecule of crystalline insulin as known amino acids. Of this, 12 per cent was present as cystine, as determined by the methods of Folin, or 8 per cent according to estimations by the Sullivan method. Freudenberg and Eyer (28) observed a rapid and irreversible inactivation of insulin by sodium sulfite, apparently due to the destruction of the disulfide linkage. They estimate that of approximately twenty sulfur atoms present in an insulin of a molecular weight of 20,000, from twelve to fifteen are present as cystine sulfur. The presence of a portion of the total sulfur as disulfide, perhaps only two of the twenty atoms, is considered essential for the activity of insulin.

METABOLISM AND NUTRITIONAL RÔLE OF THE AMINO ACIDS CONTAINING SULFUR

Although *dl*-cystine was oxidized by the white rat (29) and its sulfur excreted by the kidneys in the form of inorganic sulfate sulfur as readily as *l*-cystine, its value for the promotion of growth of the young white rat was only from one-third to two-thirds that of the natural isomer. This would suggest that the organism may be able to resolve *dl*-cystine, utilize the *l*-form for growth, and excrete the products of oxidation of the *d*-form. Confirmation of this is afforded in part by the demonstration (30) that *d*-cystine could not be utilized for purposes of growth by the white rat in lieu of the naturally occurring levo enantiomorph.

A detailed presentation of the preliminary data discussed in this *Review* in 1932,² in which it was shown that *dl*-methionine was capable of unmistakably stimulating growth in albino rats on a basal diet poor in cystine, is now available (31). That the effect of the sulfur-containing amino acids on growth promotion is specific was shown by the ineffectiveness of other amino derivatives of propionic acid (serine, alanine) in increasing the rate of growth under similar conditions. Further confirmation of this metabolic interrelationship between cystine and methionine is given in a preliminary communication from Barger's laboratory (32) in which, with a different type of basal cystine-deficient diet, *dl*-methionine was shown to be nearly as effective in improving the rate of growth as was cystine. It will be recalled that the nutritive value of casein has always been difficult to understand in view of its extremely low content of cystine (0.3 per cent). Sherman and Woods obtained values in their biological assay of casein equivalent to a content of from 1.3 to 2.5 per cent of "cystine (or cystine plus nutritionally equivalent sulfur-containing radicles)." Since it is now known that casein contains a relatively high percentage of methionine [3.25–3.53 per cent, according to Baernstein (7)], it is possible to interpret such experiments as those of Sherman and Woods on the basis of an interrelationship between cystine and methionine. Further evidence of similar metabolic rôles of these two amino acids has been obtained in the study of the mercapturic acid synthesis, as shown by changes in the partition of urinary sulfur, after the administration of monobromobenzene to dogs on diets containing protein of variable cystine content (33) with and without the addition of cystine or methionine. *dl*-methionine and *l*-cystine had similar effects on the elimination of urinary nitrogen (sparing action) and in increasing the excretion of organic sulfur after ingestion of monobromobenzene, indicating a utilization in mercapturic acid synthesis. These effects appeared to be specific for the sulfur-containing amino acids since they were not obtained when glycine was fed with the bromobenzene. In explanation of the action of methionine, it is suggested that "cystine and methionine may have some common product of intermediary metabolism, which is essential for the normal function of the organism, and that, when methionine is supplied by the diet, the cystine present is thereby made available for the detoxication of the benzene derivatives."

² Lewis, H. B., *Ann. Rev. Biochem.*, 1, 172 (1932).

Jackson and Block (31) have been led to somewhat similar conclusions as to the relation between the two amino acids in their attempts to explain the apparent interchangeability of cystine and methionine for purposes of growth. The sulfur distribution of the urine of rabbits after oral administration of monobromobenzene (34) indicated that the reactions by which the monohalogen derivatives of benzene are metabolized in the organism in the rabbit are probably similar to those in the organism of the dog.

In paired-feeding experiments with young white rats on cystine-deficient diets, the cystine content of the hair was limited by the cystine content of the diet (35). In microscopic studies of such hairs of low cystine content, the proportion of cortex, representing the completely keratinized part of the hair (according to the studies of Barritt and King),^a was much less than in normal hair, while the proportion of air cells and medullary cells, consisting of sulfur-poor substances, was correspondingly greater. It was impossible to produce a superkeratinized hair by feeding excessive amounts of cystine; the addition of cystine to a diet possessing no cystine deficiency produced no definite effect upon the composition of the hair.

In striking field experiments on the Queensland downs (36, 37), the wool clip of lambs was increased over 35 per cent by supplementing the protein-deficient pasturage with blood meal, a protein material of high cystine content (2.7 per cent). The recovery, in increased weight of fleece, of the cystine of the supplement was approximately 40 per cent. The source of the cystine made available for wool production has been the subject of much speculation (37, 38, 39) in view of the low cystine content of pasturage (23, 24). It is suggested that intestinal micro-organisms may play an important rôle in the conversion of non-cystine sulfur (inorganic sulfates?) into cystine, which becomes available after the death of the organism and the autolysis of the cell (38, 39). The recent studies on the metabolic interrelationship of cystine and methionine (31, 32) must be considered in subsequent work.

CYSTINURIA

Lewis (40) has listed and reviewed the cases of cystinuria reported in the period from 1921 to 1931 and has discussed critically

^a Lewis, H. B., *Ann. Rev. Biochem.*, 1, 172 (1932).

the recent developments in the study of this interesting error of metabolism. The importance of the chemical examination of the urine for the presence of cystine has been emphasized (41, 42). Meyer (41) studied a case of cystinuria in a woman eighty-seven years of age (the oldest cystinuric patient recorded in the literature). The incidence of cystinuria in healthy college students has been studied (42) by the chemical examination of more than 10,000 urines by the cyanide-nitroprusside and Sullivan tests for cystine. Acute cystinuria (frequent cystine crystals in urine) was observed in four individuals. A number of individuals were also studied in whose urines cystine crystals never were found but who excreted considerable amounts of cystine, as evidenced by the above-mentioned chemical tests and by the presence of an abnormally high percentage of organic sulfur in the urine.

NON-PROTEIN SULFUR OF BLOOD

As a rule, the inorganic sulfate sulfur of human blood serum (normal range, 0.8-1.7 mg. per cent as sulfur) was increased in early renal insufficiency (43) before either creatinine or urea and usually before the excretion of phenolsulfonphthalein was lowered. The inability of the kidneys to concentrate urine more closely paralleled the increased sulfate of the serum than did any other of the tests for renal function studied. Somewhat lower values for the inorganic sulfate sulfur of normal human serum (0.1-0.5 mg. per cent as sulfur) were found by Anderson and Tompsett (44), who observed increases in inorganic sulfate sulfur in normal pregnancy (from two to three times the normal values). It is pointed out that it is difficult to explain this increase during normal pregnancy unless it be assumed that, relative to nitrogen, inorganic sulfate is a more difficult substance to excrete and that, therefore, its retention may be an earlier indication of renal impairment than is afforded by the study of the non-protein or urea nitrogen. This explanation would be in harmony with the work just discussed. In eclampsia, the increases in inorganic sulfates of the serum were much more marked.

Hayman and Johnston, in a study of the concentration of sulfates by the kidney (45), also found high values for the inorganic sulfates of normal sera (1.0-2.1 mg. per cent as sulfur). Ultrafiltration experiments demonstrated that all the sulfates of blood were present in filterable form. Unusually high values for inorganic sulfate S of

serum were observed in certain cases of nephritis (e.g., 11.1, 18.4, and 31.5 mg. per cent as sulfur).

Examination of the non-protein sulfur of the human red blood corpuscle (46) has shown a concentration from 4.85 to 5.66 mg. per cent of non-diffusible sulfur. The diffusible non-protein sulfur of the corpuscles averaged about 55 per cent of the corresponding values for the plasma. It is possible to calculate the non-diffusible non-protein sulfur of the corpuscles from the total non-protein sulfur of serum and of whole blood if the percentage by volume occupied by the corpuscles is also known. The correspondence between the values thus calculated and those obtained on direct analysis is satisfactory.

GLUTATHIONE AND SULPHYDRYL COMPOUNDS

Glutathione has been isolated from potato tubers (47) treated with ethylene chlorohydrin (to break dormancy) and planted for five days. From 1.5 liters of press juice, 870 mg. of the cuprous salt of glutathione were obtained. According to the author, "this is believed to be the first isolation of glutathione from a seed plant." Investigation of various commercial tissue preparations used in the treatment of pernicious anemia and of fresh pig gastric mucosa led to the conclusion (48) that glutathione enters into the composition of anti-anemic substances, and that, in the commercial products, it appears mostly in the oxidized form.

The preparation of a crystalline copper salt of oxidized glutathione (yeast), which differed from the amorphous compound previously described in solubility and copper content, has been reported (49). Pirie (50), however, has cited evidence which indicates that there is no reason to assume the existence of a cuprous derivative of oxidized glutathione, insoluble in weak acids. The chief crystallographic data for glutathione (reduced) have been made available (50).

The search for an entirely satisfactory method for the determination of glutathione still continues. The most important methods, the iodometric and colorimetric, have been variously modified (51, 52, 53). An important factor in the iodometric titration, namely, hydrogen ion concentration, has been emphasized anew. The use of sulfosalicylic acid (53) and of trichloroacetic acid of lower concentration than that formerly used (51) as protein precipitants is recommended.

The estimation of glutathione by the determination of labile sulfur in blood filtrates has also been suggested (51), but exact details of the method are not adequately worked out.

The glutathione content of normal human blood, as determined colorimetrically (Mason), ranged from 14.7 to 38.8 mg. per cent, with average values of 21.3 (males) and 22.7 (females) mg. per cent, respectively (52). These values are somewhat lower than those usually obtained by other methods, e.g., from 25 to 41 mg., with an average of 34 mg., by Woodward and Fry (53). The data concerned with the glutathione content of blood and tissues and its variation in different physiological, experimental, and pathological conditions are so extensive as to make a review in limited space unprofitable. Lack of a specific method by which to differentiate between glutathione and other sulfhydryl compounds (if such exist) in blood and tissues makes it difficult to interpret the data. When the exact rôle of glutathione is more clearly known, it may then be possible to review and evaluate the results of experimentation satisfactorily.

Schelling (54) obtained only slight rises in the glutathione content of the blood after oral or intravenous administration of from 0.5 to 1.0 gm. of reduced glutathione to dogs. In confirmation of earlier results of Hele and Pirie, the sulfur of the glutathione molecule was easily oxidized and rapidly excreted by the kidneys as sulfate sulfur. No glutathione could be detected in the urine after subcutaneous injection of 1.0 gm. of glutathione into fasting dogs (55) nor was there any clear-cut effect on endogenous protein metabolism. The addition of glutathione to autolyzing liver, spleen, or kidney did not alter the rate of autolysis (55).

Oxidized glutathione activated amylases (56). The data as to the activating action of cysteine and glutathione on the enzyme arginase are conflicting (57, 58, 59), both activation and depression of activity having been reported.

CONJUGATED SULFURIC ACIDS

Hele (60), in important studies on the origin of urinary ethereal sulfates, has shown the availability of sodium sulfate administered either orally or subcutaneously for the synthesis of the ethereal sulfates from indole or phenol and has proved that a portion at least of the endogenous sulfur used for this synthesis may be replaced by the sulfate ion of exogenous origin. Further evidence in support of the

theory that ethereal sulfate of endogenous or exogenous origin is synthesized by direct union of the sulfate ion with phenolic or related compounds is afforded by this work. The increased urinary excretion of ethereal sulfates after oral administration of isobarbituric acid to the dog has been confirmed (61). However, the complete disappearance of organic ("neutral") sulfur from the urine under these conditions was not observed (61).

TOTAL SULFUR AND SULFATE SULFUR IN BIOLOGICAL MATERIAL—METHODS

A turbidimetric method for the determination of small amounts of sulfur has been proposed by Kemmerer and Boutwell (62), who have reported a series of analytical data, obtained by the application of this method to foodstuffs. An improved gravimetric micro-procedure for estimation of sulfur (63) has been applied to the estimation of the total sulfur of milk and tissues and of the non-protein sulfur of the blood and other body fluids.

The conditions for the precipitation of sulfates by barium chromate and the titration of the chromic acid liberated iodometrically have been determined for materials of biological importance (64). The method is adapted particularly to the determination of total sulfur, although a procedure for the partition of sulfur in human urine has also been suggested. The method of Lang,⁴ which involves also the determination of excess chromic acid, has been criticized because of its failure to remove phosphates which also precipitate barium from solutions of barium chromate and its neglect of the possible reducing action of such urinary constituents as uric acid (65).

The methods of Hubbard and of Wakefield for the determination of inorganic sulfate sulfur in blood have been modified by Power (66). In the new procedure, the sulfates are precipitated with benzidine; the benzidine in the precipitate is oxidized by potassium dichromate and the excess dichromate determined by iodometric titration.

It is regretted that limitations of space available have necessitated omission of the discussion of many papers of interest. It is hoped that some of these subjects may be treated in a subsequent issue of this *Review*.

⁴ *Biochem. Z.*, 213, 469 (1929).

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THE CHEMISTRY AND METABOLISM OF THE NUCLEIC ACIDS, PURINES, AND PYRIMIDINES*

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THE CHEMISTRY OF NUCLEIC ACIDS AND THEIR DERIVATIVES¹

In recent years, Levene and his co-workers have been engaged in a study of the arrangement of the component parts of the nucleotides derived from yeast nucleic acid. Of special interest in this connection is a knowledge of the properties and the structure of the ribose-phosphoric acids. Until recently, inosinic acid was the only nucleotide from which it had been possible to obtain ribosephosphoric acid. Levene & Dmochowski (2) have now found that xanthyllic acid, which is obtained from guanylic acid, is partly hydrolyzed on standing in aqueous solution, with the formation of ribosephosphoric acid. Thus, Levene & Harris (3) were able to compare the properties of two ribosephosphoric acids obtained from different sources. It was found that the two substances differ in several respects. By comparing the rates of hydrolysis of the phosphoribonic acids derived from them, they observed that the compound obtained from xanthyllic acid was hydrolyzed twice as fast as the one derived from inosinic acid. Differences were also noticed in the optical changes occurring in the course of lactone formation. Inasmuch as in the ribosephosphoric acid obtained from inosinic acid the phosphoric acid group is linked to carbon atom 5, as shown by previous work (4), Levene & Harris postulate that in xanthyllic acid the phosphoric acid is attached either to carbon atom 2 or 3 of the ribose, since position 5 is free, and carbon atom 4 is engaged in the ring structure.

Levene & Tipson (5) have investigated the structure of adenosine. This nucleoside was completely methylated by means of dimethyl sulfate and alkali. On hydrolysis, the methylated product gave monomethyl adenine and 2,3,5-trimethylribose. This sugar was found to have a furanoid structure. They assign, therefore, to adenosine a

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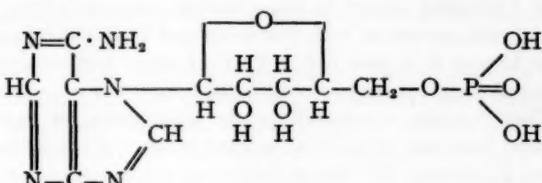
¹ For an exhaustive review of the chemistry of nucleic acids the reader is referred to the recent monograph by Levene & Bass (1).

furanoside structure. The same method was employed by these workers (6) in the case of guanosine. This nucleoside was also found to be a ribofuranoside.

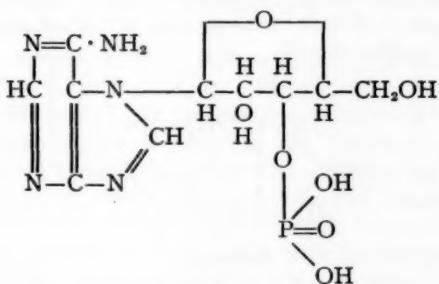
In order to obtain information regarding the structure of the ribodesose nucleosides present in the thymus nucleic-acid molecule, Levene & Cortese (7) have prepared theophylline-*d*-glucodesoside and studied its properties. A synthesis of theophylline-*l*-arabinoside is reported by Pryde & Williams (8). The study of this nucleoside leads them to believe that it has a pyranoid structure.

Soon after the discovery of adenylic acid in muscle by Embden & Zimmermann (9), Schmidt (10) reported that this compound was deaminized by muscle pulp, whereas yeast adenylic acid was not attacked. Embden & Schmidt (11) found the two nucleotides to differ in their physical and chemical properties. Steudel & Wohinz (12) have attempted to elucidate the structure of the two substances by determining the yield of pentose formed on hydrolysis. It was found that, whereas yeast adenylic acid yielded from 60 to 75 per cent of the theoretical amount of furfural, inosinic acid, derived from adenylic acid, gave only 8 to 11 per cent. Inosine, obtained from inosinic acid, gave on hydrolysis from 63 to 70 per cent of furfural. In the light of these findings the conclusion is drawn that the different location of the phosphoric acid group is the cause for the differences reported between yeast adenylic acid and muscle adenylic acid. Klimek & Parnas (13) have attacked the same problem from a different angle. Embden & Zimmermann had found that muscle adenylic acid was precipitated by a mixture of copper sulfate and calcium hydroxide. This fact led Klimek & Parnas to investigate the behavior of yeast adenylic acid toward the same reagent. They found that it was not precipitated. This means that muscle adenylic acid forms complex copper compounds, yeast adenylic acid does not. That this behavior of yeast adenylic acid is to be attributed to the location of the phosphoric acid group in its molecule, becomes apparent from the fact that the corresponding nucleoside, adenosine, does react with the copper reagent. Following up these observations, Klimek & Parnas studied the behavior of the two nucleotides toward boric acid. According to Böseken (14), only polyhydroxy compounds, containing two vicinal hydroxyl groups in the cis position, will increase the conductivity of boric acid. It was found that the ribose in muscle adenylic acid contains two such hydroxyl groups, whereas yeast adenylic does not. On the basis of these observations, and the work

of Levene and associates (4) on the structure of inosinic acid, Klimek & Parnas assign to muscle adenylic acid and to yeast adenylic acid the following formulae:



Muscle adenylic acid



Yeast adenylic acid

An alternative formula for yeast adenylic acid, containing the phosphoric acid group attached to carbon atom 2 of the ribose, is also conceivable since the exact location of this group is not yet known.

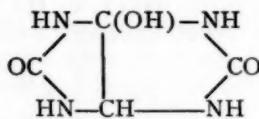
Kobayashi (15) has compared the rates of hydrolysis of the yeast nucleotides—guanylic acid, adenylic acid, cytidylic acid, and uridylic acid. He finds that, of the four nucleotides, guanylic acid is the least resistant to hydrolysis. Adenylic acid is next in resistance. In the case of the pyrimidine nucleotides, the liberation of the phosphoric acid takes place with great difficulty.

Stearn (16) has carried out conductometric titrations of methyl violet with sodium nucleate and sodium gelatinate. Titrations were

made in both directions, i.e., the gelatin and the nucleic acid were titrated with the dye, and also the dye was titrated with the gelatin and the nucleic acid. In all cases definite equivalence values were found, which were independent of the direction of the titration. The apparent equivalent weight of yeast nucleic acid, according to their data, is in good agreement with that calculated from the formula proposed by Levene & Simms (17). Fürth, Leipert & Kurokawa (18) have studied the properties of certain alkaloidal salts of yeast nucleic acid. They describe a method for the preparation of pure yeast nucleic acid from the commercial product by way of the brucine salt.

Fisher & Johnson (19) report that convicine, a pyrimidine nucleoside occurring in vetch, is a hexoside of 4-imino dialuric acid.

Several interesting papers on the oxidation of uric acid *in vitro* have appeared. Frèrejacque (20) has studied the effects of manganese dioxide in the presence of certain amines. He finds that uric acid under these conditions forms ureides, which are assumed to be derivatives of isoallantoin (I).



I. Isoallantoin

These observations point to isoallantoin as being one of the first intermediates in the oxidation process: uric acid → allantoin. Zylbertal (21) has investigated the oxidation of uric acid in the presence of various types of charcoal. He finds that adsorbed uric acid is oxidized in one of two ways, according to the type of charcoal used. One type, containing metallic impurities, will oxidize uric acid, but this oxidation is inhibited by cyanide. The other type is metal-free, and oxidation is not checked by cyanide. A third type of charcoal will adsorb uric acid, but no oxidation takes place. More (22) has studied the action of iodine on uric acid in an alkaline medium in the presence of ammonium salts. He was able to identify diamino-oxalyldiurea as one of the end-products of the oxidation. Wieland & Macrae (23) find that hydrogen peroxide at pH 8.0 and at 37° converts uric acid into ammonia, urea, oxalic acid, carbonyldiurea,

cyanuric acid, and allantoin. The last product was obtained in greatest quantity. Schuler & Reindel (24) have studied the oxidation of uric acid by potassium permanganate in alkaline solution. They isolated from the reaction mixture the silver salt of a substance to which they assign the structure of a hydroxy-acetylene-diurein carboxylic acid.

Johnson & Flint (25) have investigated the action of ozone on thymine, uracil, and other pyrimidines. Uracil and ozone interact at ordinary temperature to form chiefly formylglyoxylurea and oxaluric acid. These findings are of biochemical interest, because oxaluric acid is known to be present in small amounts in the urine. Thymine underwent a more complete breakdown, giving formyl urea. The effects of dilute acids and of light energy on thymine glycol have been studied by Johnson, Baudisch & Hoffmann (26). Methods for the synthesis of orotic acid have been described by Johnson & Schroeder (27) and by Hilbert (28).

Hill (29) has continued his studies on the properties of dialuric acid. He finds that glycine, alanine, valine, glutamic acid, and phenylalanine are oxidized by air in the presence of dialuric acid at pH 7.0 to the corresponding lower aldehydes. It is assumed that dialuric acid is converted into alloxan, which then oxidizes the amino acid, and is itself again reduced to dialuric acid, so that the process is continuous. These observations indicate a possible function of uracil in the oxidation processes in the tissues. The experiments of Cerecedo (30) suggest isodialuric acid as being an intermediate in the metabolic breakdown of uracil, and it is a well-known fact that isodialuric acid is converted into dialuric acid in an alkaline medium.

Heyroth & Loofbourow (31) have determined the ultraviolet absorption spectra of uracil, dichloromethylpyrimidine, adenine, and thymus nucleic acid, and studied the effects of ultraviolet irradiation upon these spectra.

METHODS

Uric acid.—Methods for the determination of uric acid in blood have been described by Benedict & Behre (32) and by Rosenthal (33). Blankenstein (34) reports the results of a comparative study of various methods for the estimation of uric acid in blood serum. Krupski & Almasy (35) claim that uric acid can be detected spectrographically in the blood and cerebrospinal fluid.

Bergami (36) offers a method for the estimation of uric acid in bile.

New procedures for the determination of uric acid in urine have been described by Christman & Rawwitch (37), Establier Costa (38), Bordley & Richards (39), Salt (40), and Barnard (41).

Methods for the estimation of uric acid in avian excrement have been developed by Suzuki & Nishizaki (42) and by St. John & Johnson (43).

By means of three histochemical reactions, Schultz & Schmidt (44) have been able to detect the various forms of uric acid present in tissues.

Allantoin.—Fosse, Brunel & Thomas (45) offer a new procedure for the determination of allantoin in blood.

Methods for the estimation of allantoin in urine have been developed by Fosse, Brunel & Thomas (46), Champagne & Mourot (47), Larson (48), Ro (49), and Allen & Cerecedo (50).

Cole, Ellett & Womack (51) have worked out a procedure for the determination of xanthine and hypoxanthine in blood. Schmidt & Engel (52) offer a method for the estimation of guanine in tissues.

Thomas (53) describes a color reaction which enables him to distinguish between yeast nucleic acid and thymus nucleic acid.

A method for the determination of nucleoprotein phosphorus in tissues has been described by Javillier and his associates (54).

PURINE METABOLISM

In his previous investigations, Lucke (55) had shown that in man from 30 to 50 mg. of uric acid are excreted daily into the intestinal canal by way of the gastric juice and the bile. He now finds (56) that the greater part of the uric acid, which thus appears in the intestines, is destroyed by bacteria, since the amount of uric acid present in the feces is very small, and apparently no reabsorption of uric acid takes place. The amount excreted into the intestines may increase in hyperuricemia, so that it is possible for the intestine to take over this excretory function to a certain extent in renal insufficiency. This also explains the disappearance of a certain amount of uric acid, when it is injected. According to Lucke, there is no necessity for assuming the existence of a uricolytic enzyme in man.

Rothmann (57) has determined adenylic acid in the blood and the bile. The average value of this nucleotide in blood is from 15 to

18 mg. per cent. He finds that there is a distinct correlation between adenylic acid and erythrocyte count. In anemia the lowest values are found, in polycythemia the highest. The daily destruction of erythrocytes explains the relatively large amount of adenylic acid, which is excreted in the bile. Part of this is probably reabsorbed. The ingestion of this nucleotide leads to a rise in the excretion of uric acid in the urine. Rothmann believes that adenylic acid plays an important rôle in the endogenous metabolism of uric acid.

Saiki, Olmanson & Talbert (58) find that uric acid is always present in the sweat in small amounts. Their data show that the uric acid content of both blood and urine rises after sweating. Melka (59) observed an increase of uric acid in the urine after profuse sweating caused by strenuous exercise. If the sweating was kept to a minimum during the exercise, less uric acid was excreted.

Terroine & Mourot (60) maintained young pigs on a high-protein diet, adequate for good growth, but deficient in inorganic salts. They observed a drop in the nitrogen retention, which was paralleled by a rise in the excretion of allantoin and purine bases in the urine. These results suggest that purine compounds may be formed from the protein in the diet. Following up these observations, Terroine, Gajaja & Bayle (61) kept growing pigs on protein-free diets and on diets containing various amounts of protein. In every case they found that a rise in the total nitrogen output was accompanied by an increased elimination of allantoin and purine bodies.

Allen & Cerecedo (62) have investigated the metabolism of guanosine and adenosine in the dog. When fed in small amounts, the two nucleotides were almost completely metabolized. It was found that about one-half of the nitrogen of guanosine appears in the urine in the form of allantoin, and one-third is converted into urea. In the case of adenosine, the same increase in urinary allantoin was observed, but practically no rise in ammonia or urea. The results obtained with guanosine seem to indicate that in the dog the end-product of purine metabolism is not allantoin alone: part of the purine molecule seems to be further broken down to urea.

PYRIMIDINE METABOLISM

Previous work dealing with the metabolism of uracil, isobarbituric acid, and isodialuric acid in the dog had been interpreted by Cerecedo

(30) as indicating that the oxidation of uracil in the animal body takes place according to the following scheme: uracil → isobarbituric acid → isodialuric acid → urea + an unknown carbon compound. The formation of formyloxaluric acid and oxaluric acid in the oxidation of uracil *in vitro* suggested that these two substances might also be intermediates in the catabolic breakdown of uracil *in vivo*. Cerecedo (63) has now found that these compounds, when fed in small amounts, are to a great extent catabolized to urea. These findings suggest that in the metabolism of uracil the sequence of reactions is: uracil → isobarbituric acid → isodialuric acid → oxaluric acid → urea + oxalic acid. Stekol & Cerecedo (64) have determined the various sulfur fractions in the urine of the dog after feeding isobarbituric acid, isodialuric acid, and formyloxaluric acid. The rise of the ethereal sulfate fraction caused by isobarbituric acid suggests that this substance is partly excreted in conjugation with sulfuric acid. The administration of these compounds produced in every case a drop in the neutral sulfur fraction. Lawrie & Pirie (65) have also observed a rise in the ethereal sulfate fraction after administration of isobarbituric acid, but they were unable to confirm the findings of Stekol & Cerecedo as regards the drop of the neutral sulfur following the feeding of this compound.

Cerecedo (66) has studied the metabolism of parabanic acid, alloxan, and alloxanthin in the dog. His findings indicate that parabanic acid is to a great extent excreted unchanged. Following the feeding of alloxan a distinct drop in the output of inorganic sulfates was noted. Inasmuch as this decrease was not compensated by the rise of any other sulfur fraction of the urine, the assumption was made that alloxan is possibly partly excreted in the bile, perhaps as an ethereal sulfate. Part of the alloxan is converted into alloxanthin. Alloxanthin seems to be partly excreted in the bile and partly eliminated in the urine unchanged.

MECHANISM OF URIC ACID EXCRETION

Wigglesworth (67) has put forward a very interesting theory regarding the mechanism of uric acid excretion in insects. He finds that the blood-sucking insect, *Rhodnius prolixus*, excretes almost all of the nitrogen in the urine in the form of uric acid, of which from 80 to 90 per cent is free. The mechanism by which the highly in-

soluble uric acid is excreted in the comparative absence of water is explained as follows: The upper part of the Malpighian tubes secretes a solution of acid urates from the blood into the lumen, while base and water are reabsorbed from the lower part of the tubes, leading to a precipitation of the insoluble uric acid. Thus there is a continuous circulation both of water and base. These findings suggest that the same mechanism may be employed in the elimination of uric acid by reptiles and birds. The probability of a circulation of water through the excretory system of these animals has long been recognized. The possibility of a circulation of base is now suggested by Wigglesworth's observations. Needham (68) points out that a similar mechanism of circulation of base and water would account for the presence of free uric acid in the allantoic liquid during the last week of incubation of the chick embryo.

Boyden (69) has studied the morphological development of the mesonephric tubules of the chick embryo, ranging from 5 to 14 days of incubation. He finds that during this period the different parts of the mesonephric tubule show different rates of growth. His observations indicate that during the period when the excretion of uric acid is increasing the secreting portion of the mesonephric tubule is the part that is growing the fastest.

THE ACTION OF ENZYMES ON NUCLEIC ACIDS AND THEIR DERIVATIVES

Klein (70) has obtained a very potent nucleotidase preparation from the intestinal mucosa of the calf. He finds that thymus nucleic acid and yeast nucleic acid behave differently toward this enzyme. Whereas in the case of the former the phosphoric acid is almost completely split off, the molecule of yeast nucleic acid is more slowly broken up, and the separation of phosphoric acid is not as complete. Further observations on the action of this nucleotidase on thymus nucleic acid are reported by Bielschowsky & Klein (71). They were able to identify guanosine, thymosine, cytidine, inosine, and xanthosine as degradation products. The same nucleosides were found by Bielschowsky (72) as breakdown products of a nucleic acid preparation obtained from pus. Yeast nucleic acid was found by Bielschowsky & Klemperer (73) to yield guanosine, inosine, uridine, and cytidine.

Levene & Dillon (74) have obtained evidence of the occurrence

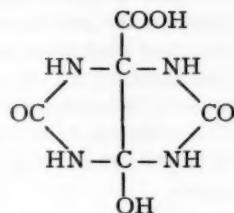
of a nucleotidase and a polynucleotidase in the gastrointestinal secretions of the dog. Attempts to separate the two enzymes were unsuccessful. Mroczkiewicz (75) has investigated the action of a nucleotide deaminase obtained from frog muscle on the adenylic acids of yeast, muscle, and pig's blood. He finds that the preparations obtained from muscle and pig's blood behave similarly, but differently from yeast adenylic acid. He concludes that the two former substances are identical.

Edlbacher & Kutscher (76) report that the nucleotidase present in liver and in tumors can be rendered more active by extracting the tissues with acetone. This treatment removes an inhibitory substance. Hydrocyanic acid, cysteine, and glutathione exert an influence on the nucleotidase similar to that of the natural inhibitor. Nucleic-acid breakdown in liver and in mouse carcinoma shows the same response to those three substances. It is also activated by the acetone treatment. These findings suggest that this nucleotidase, like peroxidase and catalase, contains a heavy metal complex. The results of these workers are in agreement with the observations of Waldschmidt-Leitz & Schäffner (77) regarding the inhibitory effects of SH-compounds on the action of Levene & Dillon's (78) nucleotidase on thymus nucleic acid.

A new phosphatase, different from all known phosphatases, which shows a specific action on adenylylpyrophosphoric acid, is reported by Jacobsen (79) to be present in the liver, and to a lesser extent in the kidney. He proposes the term "adenylylpyrophosphatase" for this new enzyme. His observations have been confirmed and extended by Barrenscheen & Lang (80).

The action of uricase has been investigated by several workers. Grymberg (81) reports a study of the kinetics of the action of uricase on uric acid. He finds that it proceeds more energetically in the presence of oxygen than of air. It requires one molecule of oxygen and one molecule of water, one molecule of carbon dioxide being liberated. With very small uric acid concentrations, the reaction tends to become one of the first order. These observations regarding the mechanism of the transformation of uric acid into allantoin are confirmed by the work of Schuler (82). This worker finds that it consists of three processes—oxidation, hydrolysis, and decarboxylation. The oxidation proceeds best at pH 8.9, the decarboxylation at pH 9.9. Thus, both processes may be experimentally separated. Schuler interprets his observations as indicating that hy-

droxyacetylene-diurein-carboxylic acid (II) is an intermediate in the reaction uric acid → allantoin.



II. Hydroxyacetylene-diurein-carboxylic acid

Ro (83) finds that uricase acts only on uric acid, and has no effect either on hypoxanthine or xanthine. The reaction velocity follows almost exactly Schütz's rule. Uricase acts best at pH 9.4 and at 45°. Oxygen is found to be absolutely essential for the action of the enzyme. The reduction-oxidation potential is increased when uric acid is acted upon by uricase, indicating that an intermediate substance of high oxidation potential is first formed, which is later converted to allantoin. These findings seem to support Schuler's assumption as regards the formation of hydroxyacetylene-diurein-carboxylic acid, since this compound would be a stronger acid than uric acid itself.

Truszkowski (84) has reported further observations on uricase. He believes that its behavior suggests a protein configuration. Its inactivation by potassium cyanide seems to indicate the presence of a heavy metal, possibly copper. Uricase is inactive below pH 7.4 and above pH 9.5. Mercuric chloride, iodine, and hydrogen peroxide in the presence of ferrous sulfate were found to inhibit the action of uricase.

Dmochowski (85) has been unable to confirm the findings of Przylecki (86) regarding the transformation of urea into uric acid by liver pulp. He finds that, instead of increasing, the purine nitrogen is diminished in the course of autolysis.

The action of *Bacterium acidi urici* and *B. aerogenes* on uric acid and on certain substituted uric acids has been studied by Hanzal & Ecker (87). They report that only uric acid is destroyed by these micro-organisms; the uric acid derivatives are not attacked.

Ro (88) offers a method for the preparation of allantoinase from

soybean meal. He finds that this enzyme acts best at pH 7.3 and at temperatures ranging from 50° to 60°.

Schmidt (89) has succeeded in separating two deaminases from rabbit liver. One is highly specific and causes the deamination of guanine and guanosine, the other deaminizes guanylic acid. He finds that, whereas cytosine and adenine are only deaminized when they are in the form of nucleosides, such combination is not necessary in the case of guanine. This different behavior of guanine enabled Schmidt & Engel (52) to work out a method for the estimation of guanine compounds in tissues.

ADENYLPYROPHOSPHORIC ACID

By extracting frog muscle with supercooled isotonic potassium chloride solution, cell-free extracts can be obtained which are very rich in glycolytic power. These extracts become inactive in a few hours, even at room temperature. For their reactivation there seem to be at least two components necessary, which together react as a co-ferment system. One of these, the autolyzable fraction, disappears from the extract on standing. The other is stable. The autolyzable component was isolated by Lohmann (90) from fresh muscle extract in the form of its barium salt. This substance, on neutral hydrolysis, was found to yield pyrophosphoric acid and adenylic acid. These observations indicate that this new compound is an adenylypyrophosphoric acid. The position of the pyrophosphoric acid group in the molecule is as yet unknown. The compound was also found independently by Fiske & Subbarow (91). According to Lohmann (92), the formation of lactic acid in muscle is brought about in the presence of a co-ferment system consisting of three components: inorganic phosphate, adenylypyrophosphoric acid, and magnesium. The deterioration of a muscle extract on standing is due to the enzymatic breakdown of adenylypyrophosphoric acid. This breakdown may proceed along two lines: it may yield inosinepyrophosphoric acid and ammonia, or adenylic acid and two molecules of phosphoric acid. Addition of adenylypyrophosphoric acid restores to the inactive muscle extract its glycolytic power. If the inactivation has not gone too far, adenylic acid will have the same effect, since such extract is able to synthesize the pyronucleotide from adenylic acid and inorganic phosphate. Inosinepyrophosphoric acid will also restore partly the glycolytic activity. Inosinic acid, however, is inef-

fective. Mozolowski, Reis & Sobczuk (93) report that the enzymatic breakdown of adenylylpyrophosphoric acid may be controlled by changing the pH of the muscle preparation. Borate buffers will produce a degree of alkalinity at which the ammonia formation is inhibited, while the pyrophosphate hydrolysis will still proceed. These workers also find that in freshly prepared muscle pulp these two processes occur simultaneously. The parallelism between ammonia formation and liberation of pyrophosphate has also been observed in muscle extract by Meyerhof, Lohmann & Meyer (94) and in human blood by Barrenscheen & Filz (95).

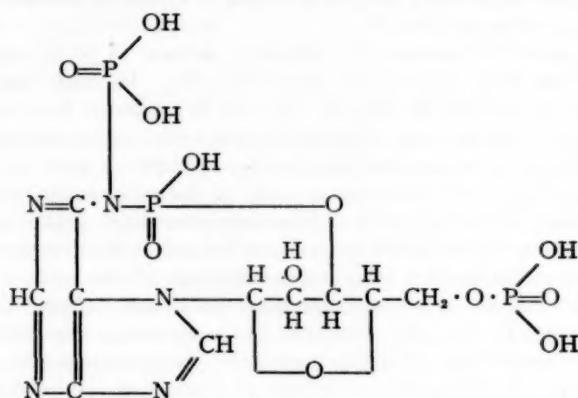
A similarity between the glycolytic process in blood and in muscle has been observed by Meyerhof (96). He finds that the enzyme preparation of rat-blood cells can be separated from its co-enzyme by ultrafiltration. The glycolytic activity can be restored by the addition of magnesium and adenylylpyrophosphoric acid.

Engelhardt (97) has made a study of the relationship between respiration and the function of adenylylpyrophosphoric acid in avian erythrocytes. He finds that in nucleated red cells a decomposition of the pyronucleotide takes place under conditions of true anaerobiosis. The fact that no such breakdown takes place under aerobic conditions suggests that the substance is continuously resynthesized through respiration. Engelhardt regards the pyronucleotide as a component of the co-enzyme system of respiration. A breakdown of adenylylpyrophosphoric acid to adenylic acid during anaerobiosis of mammalian muscle has also been observed by Cori (98).

THE STRUCTURE OF ADENYLYL PYROPHOSPHORIC ACID

Barrenscheen & Filz (99) have attempted to establish the structure of adenylylpyrophosphoric acid. They find that on hydrolysis with hydrochloric acid only very small amounts of pentose are formed. In this respect the substance resembles muscle adenylic acid. On the other hand, the deamination with nitrous acid proceeds more slowly in the case of the pyronucleotide. The products of this reaction were found to be phosphoric acid and inosinic acid. Inosine-pyrophosphoric could not be detected, whereas Lohmann (92) maintains that it is formed. They also observed that the deaminase, which Schmidt (10) found to be specific for muscle adenylic acid, does not attack adenylylpyrophosphoric acid. However, the deaminase of Schmidt in conjunction with the phosphatase, discovered by Jacob-

sen (79), was found to cause the liberation of ammonia and phosphoric acid from the pyronucleotide. These findings are interpreted as indicating that the amino group of adenylypyrophosphoric acid is not free. Two phosphoric acid groups are attached to it, forming an iminopyrophosphate combination. One of these phosphate groups is probably linked to one of the carbon atoms in the pentose. The following formula is tentatively proposed for the pyronucleotide:



Adenylypyrophosphoric acid

Lohmann (100) disagrees with Barrenscheen & Filz in several respects. In the first place, he maintains that inosinepyrophosphoric acid may be obtained on treatment of adenylypyrophosphoric acid with nitrous acid. In fact he describes a method for the preparation of the inosine compound by means of this reaction. He also points out that under certain conditions the liberation of ammonia from adenylypyrophosphoric acid may take place more rapidly than the hydrolysis of the two easily hydrolyzable phosphate groups. Further evidence in contradiction to their views is seen in the fact that adenylypyrophoric acid, like muscle adenylic acid, forms a complex copper salt. Klimek & Parnas (13) have pointed out that this behavior of muscle adenylic acid suggests that two vicinal hydroxyl groups of the pentose are in the *cis* position. If this argument holds, two of the

hydroxyl groups in the pyronucleotide must be in the *cis* position, whereas in Barrenscheen & Filz's formula one of these hydroxyl groups is esterified by a phosphate group.

THE FUNCTION OF ADENYLPYROPHOSPHORIC ACID IN MUSCLE

As to the function of adenylypyrophosphoric acid in muscular activity, recent observations of Meyerhof & Lohmann (101) and of Meyerhof (102) show that the breakdown of this substance to orthophosphate, ammonia, and inosinic acid, is accompanied by the liberation of energy. This energy, according to these investigators, is utilized for the synthesis of creatinephosphoric acid. The phosphoric acid which is liberated serves for the esterification of carbohydrate before it is broken down to lactic acid. The energy set free by the breakdown of hexosephosphate to lactic acid is in its turn utilized for the resynthesis of adenylypyrophosphoric acid. A possible function of this compound in cellular activities is suggested by the findings of Deuticke (103). This worker reports the occurrence of specific dehydrogenases in plant seeds, which reduce methylene blue only in the presence of adenylypyrophosphoric acid.

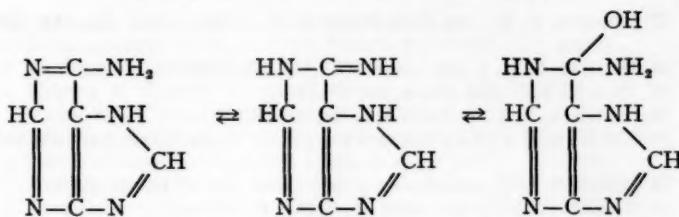
THE COZYMASE RÔLE OF NUCLEOTIDES IN ALCOHOLIC FERMENTATION

Another field of investigation has been opened up by the studies of H. von Euler and his co-workers on the chemistry of the cozymase of alcoholic fermentation. Euler (104) has reviewed these investigations in a recent monograph. Further information on the properties of cozymase is given by Euler & Myrbäck (105). They describe a method for obtaining a highly active cozymase preparation. Analysis of this product shows it to be very closely related to muscle adenylic acid. Cozymase is defined by these workers as that substance which brings about fermentation in an otherwise inactive mixture of sugar, phosphate, zymophosphate, apozymase, and magnesium salts. Studies of the rate of hydrolysis of a highly purified cozymase preparation by Myrbäck & Euler (106) indicate that the substance belongs to the same group as muscle adenylic acid, but is different from yeast adenylic acid. Molecular weight determinations of highly active cozymase preparations, carried out by Myrbäck & Euler (107), gave values which agree closely with those of adenylic

acid. Deamination of cozymase by nitrous acid yielded inosinic acid, which is also given by muscle adenylic acid.

Lohmann (108) has found that adenylypyrophosphoric acid will not only restore glucolytic activity to inactive muscle extract, but will also activate apozymase. Euler & Myrbäck (109) report observations which are not in agreement with Lohmann's findings. They maintain that adenylypyrophosphoric acid will show under the best conditions only 10 per cent of the activity of the purest cozymase preparations. They also report that muscle adenylic acid shows no measurable cozymase action. That adenylypyrophosphoric acid has no specific activating effect on apozymase is also shown by the observations of Nilsson & Euler (110). They find that different preparations of adenylypyrophosphoric acid vary considerably in their power of activating apozymase, and that after purification by repeated precipitation as mercury, lead, and barium salts, they may lose their activity altogether. On the other hand, Euler & Nilsson (111) have confirmed the observations of Lohmann (112) to the effect that the cozymase of yeast will restore the glucolytic power to inactive muscle extracts. But, whereas Lohmann claims that adenylypyrophosphoric acid is more active than cozymase in this respect, these workers find that the effect of the two substances is about the same. Myrbäck, Euler & Hellström (113) have determined the ultraviolet absorption spectra of cozymase, adenylypyrophosphoric acid, muscle adenylic acid, yeast adenylic acid, inosinic acid, and guanosine. The spectra of cozymase and the other adenine derivatives all show a maximum between 2580\AA and 2600\AA . The similarity of the spectra given by the various adenine compounds indicates that this method is not applicable to the study of such fine structural differences as those shown by yeast adenylic acid and muscle adenylic acid.

An interesting theory regarding the function of cozymase has been put forward by Zuckerkandl & Messiner-Klebermass (114). They find that yeast which has been poisoned by iodoacetic acid or sodium fluoride, and is thus unable to ferment glucose, may be reactivated by the addition of certain amines. The function of these substances is explained as follows: the amines are converted into imines; these interact with glucose, whereby a molecule of water is split off from the sugar; thus, the glucose molecule is converted into a form which will easily undergo cleavage. If these considerations are applied to adenine, we should have the following sequence of reactions:



However, as long as the exact structure of cozymase is not known, this theory is to be regarded as mere speculation.

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CARBOHYDRATE METABOLISM*

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In their review last year, Shaffer & Ronzoni¹ presented a comprehensive and clear picture of the major biochemical problems of the day as related to carbohydrate metabolism and established the necessary connections with previous experimental work in this field. They have thus paved the way and made much easier the task of future surveys.

The present article is confined to publications which appeared in the year 1932. Owing to the limited space available, only 50 per cent of the total number of papers read and abstracted could be included in this survey. Under such restricted conditions the selection of papers for review becomes an increasingly difficult task.

Though many notable results have been achieved, there has been no radical departure from previous concepts, except perhaps in the position of methylglyoxal as an intermediary of carbohydrate metabolism.

GLYCOGEN IN LIVER AND MUSCLE

a) *Relation of water to glycogen storage.*—The statement found in textbooks that 1 gm. of glycogen leads to the retention of about 3 gm. of water in the liver was re-investigated by Puckett & Wiley. They found in experiments on rats that the water content of the liver remained constant (70.3 ± 0.7 per cent), in spite of variations in the glycogen content from 0 to 7.6 per cent. The authors concluded that glycogen stores the same amount of water as the other liver solids, i.e., about 2.4 gm. of water per gm. of solid. Mackay & Bergman, who performed similar experiments on young rabbits, found no change in the water content of the liver over a range of glycogen of 0.2 to 13.3 per cent. Holmquist carried out an extensive investigation of the same problem on hedgehogs, rabbits, rats, mice, and

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pigeons. The water content of the liver was found to be surprisingly constant in the different species regardless of wide variations in the glycogen content. The foregoing data are in agreement in showing that, when glycogen is deposited (or lost) in the liver, water is retained (or lost) in proportional amounts, so that there occurs practically no change in the water content of the liver cell. Bridge & Bridges in commenting on these papers think it unjustified to define an exact mathematical relationship between glycogen and water content of the liver.

b) Cyclic changes in liver.—Experiments by Higgins, Berkson & Flock, though undertaken for another purpose, also fail to show a significant change in the water content with variations in glycogen from 0.8 to 6.5 per cent. One year ago Ågren and co-workers described cyclic changes in the glycogen content of the liver of rabbits, rats, and mice and claimed that these changes were largely independent of food intake. Glycogen accumulated in the liver during the night, even though the animals were fasting, and disappeared again to some extent the next morning. Higgins *et al.* allowed rats to feed and drink on several consecutive days between 9:00 and 11:00 A.M. After the last feeding period, groups of rats were killed and analyzed every 2 hours for 46 hours. In the first 24 hours after feeding, the curves representing changes in weight and in the glycogen and protein content of the liver showed two peaks, one occurring at 7:00 and the other at 11:00 P.M. These cyclic changes were definitely related to the food intake, because during the second 24 hours, when the animals were in a truly fasting condition, all cyclic changes had disappeared. There was, in particular, no deposition of glycogen during the night, as had been claimed by Ågren and co-workers. The nature of the diet, which is unfortunately not stated in this paper, is undoubtedly a factor of major importance in the interpretation of these findings. It may be assumed that diets of different composition will yield very different curves for the various factors investigated.

c) Glycogen formation.—Stöhr (1) tested various three-carbon compounds for their ability to form glycogen in liver and muscle of rats. Three hours after feeding 100 mg. of methylglyoxal per 100 gm. of body weight the liver glycogen was 0.52 per cent, as compared to 0.12 per cent for the controls. Larger doses of methylglyoxal (150 to 200 mg. per 100 gm. body weight) were toxic and caused a decrease in glycogen. Three hours after feeding 100 to

185 mg. of pyruvic acid (as sodium salt) per 100 gm. body weight the average liver glycogen was 0.77 per cent and a similar value (0.79 per cent) was obtained after feeding 200 to 300 mg. of *dl*-glyceric aldehyde per 100 gm. body weight. Glyceric aldehyde was found to be absorbed more slowly than methylglyoxal; both substances (2) were found to cause a fall in the alkali reserve of the blood, which was ascribed to lactic-acid formation. Glyceric aldehyde, methylglyoxal, and dihydroxyacetone were tested for their ability to form muscle glycogen, but only the last-named substance was able to do so. A reaction product of methylglyoxal and acetoacetic acid, ketol [3-hydroxy-acetonyl-acetone ($C_6H_{10}O_5$)], was also investigated by Stöhr & Henze. *In vitro* and possibly also *in vivo* this compound is split into 2 molecules of methylglyoxal (or pyruvic acid); it was found to behave like methylglyoxal in its ability to form liver glycogen but not muscle glycogen. Acetol, which is $CH_3 \cdot CO \cdot CH_2OH$, did not form liver glycogen in rats (Stöhr & Müller).

Ponsford & Smedley-Maclean fed rats on a diet high in fat and low in carbohydrate. Two grams of the ammonium salts of succinic, fumaric, malic, or acetic acids were added to the basal diet for 3 days. The first three acids formed liver glycogen, while acetic acid did not. Eckstein observed glycogen formation in the liver of the rat after oral administration of propionate, while butyrate, valerate, and caproate were negative. It was ascertained that the acids were actually absorbed from the gut. Xylose, when fed to rats, formed no significant amount of glycogen in the liver (Miller & Lewis).

Long & Horsfall determined glycogen formation in muscle of the decapitated, eviscerated cat following injection of sarcolactic acid. It had been shown previously that when glucose is injected into such a preparation, the muscle glycogen remains unchanged, while the injection of glucose plus insulin leads to the deposition of muscle glycogen, an observation which was confirmed by Long & Horsfall. In five experiments with lactic acid (injected, $\frac{1}{2}$ to $\frac{3}{4}$ neutralized) there was a rise in muscle glycogen amounting to 50 to 100 mg. per cent; in four experiments the rise was less than 50 mg. per cent and in five experiments there was either no change or a slight fall in muscle glycogen. In six experiments glucose and lactic acid were injected, the glycogen rising 100 to 160 mg. per cent in three and remaining unchanged in the other three cases. In five experiments glucose, insulin, and lactic acid were administered with the result

that more glycogen was deposited than when only glucose and insulin were given. This is shown by the fact that in the experiments with glucose plus insulin 50 per cent of the injected glucose was accounted for as glycogen, while in the experiments in which lactate was added 95 per cent was thus accounted for. Hence it was concluded that part of the lactate was deposited as muscle glycogen. Lactate disappeared in these preparations at the rate of 240 mg. per kilo per hour; glucose disappeared at an equal rate; addition of insulin raised glucose utilization to 410 to 570 mg. per kilo per hour but did not increase utilization of lactate.

d) Effect of anesthetics.—All the anesthetics investigated by Murphy & Young, namely chloroform, amytal, luminal, dial, numal, pernoctone, and chloralose, lowered the liver glycogen. The frequent sampling of the liver necessary in these experiments may be an additional factor making for a loss of glycogen. There was also a rise in blood sugar. Amytal had the smallest effect on the liver glycogen, chloralose on the blood sugar. The authors conclude that experiments carried out under the above-named anesthetics involving taking of liver samples are unsatisfactory. Major & Bollman, on the other hand, concur with several previous observers that amytal has little if any effect on muscle glycogen, whereas ether causes a marked diminution when administered for $2\frac{1}{4}$ hours.

e) Starvation, phlorhizin, pancreatectomy.—Experiments on three dogs fasted for three weeks and exercised frequently on a treadmill were reported by Dann & Chambers. Samples of liver and muscle were removed during the fast and following the ingestion of 25 gm. of glucose. Glycogen increased in both liver and muscle after the carbohydrate meal, and from this it was concluded that there occurs no derangement of glycogen formation during fasting. The decreased tolerance of fasting dogs for carbohydrate seems to be due mainly to a diminution in carbohydrate oxidation.

Phlorhization has been compared to prolonged fasting in its effect on carbohydrate utilization, and it is of interest, therefore, that administration of glucose to completely phlorhizinated dogs led to the deposition of significant amounts of liver and muscle glycogen, as shown by Major. Even the pancreatectomized dog, according to the experiments of Major & Mann (1), is capable of some glycogen formation in liver and muscle, when large amounts of glucose are injected. Pancreatectomized dogs were first treated with insulin until the wound was completely healed. Insulin injections were omitted

for five days prior to the experiments. It may then be assumed that the tissues are free of insulin, especially since according to the investigations of Best and collaborators very little, if any, insulin is present even in the extra-pancreatic tissues of the normal animal. Eight negative and six positive experiments are reported for muscle, the negative experiments being explained by the fact that the animals, which were under the influence of an anesthetic, began to shiver. Five negative and ten positive experiments are reported for liver. Though no blood-sugar values are given in the paper of Major & Mann (1), it may be inferred from the large amounts of glucose injected that the blood-sugar levels at which glycogen synthesis occurred were very high.

METABOLISM UNDER DIABETIC CONDITIONS

Chambers, Kennard & Pollack made a study of the respiratory metabolism of exercise and recovery in depancreatized dogs. Muscular activity was induced by electrical stimulation under amytal anesthesia. The respiratory quotient rose slightly during the exercise period, but this was attributed to changes in the CO₂ content of the blood coincident with the rise in blood lactic acid rather than to oxidation of carbohydrate. During the recovery period the respiratory quotient often fell to subnormal values, suggesting a retention of CO₂. All the dogs contained muscle glycogen, so that the absence of carbohydrate oxidation could not be attributed to lack of this fuel. The conclusion was reached that fat is the fuel of exercise and recovery in depancreatized dogs. Ring & Hampel observed an average respiratory quotient of 0.70 in depancreatized cats kept for at least two days without insulin. The oxygen consumption was 42 per cent above that of normal cats. In contrast to skeletal muscle and to the animal as a whole, brain seems to have a respiratory quotient of unity under all conditions, as shown by experiments on depancreatized and phlorhizinized dogs and after insulin, pituitrin, and adrenalin injections. Himwich & Nahum conclude from their experiments that brain always oxidizes carbohydrate.

Page & Young report that injections of phosphatides do not change the dextrose-nitrogen ratio in the urine of phlorhizinized dogs, while glucose injection is followed by excretion of adequate amounts of extra sugar. Hence they conclude that a conversion of

fatty acids to sugar does not take place in the phlorhizinized animal. In 1926 Wertheimer claimed that phlorhizinized dogs, with a fatty liver, are more resistant to insulin than normal dogs. He assumed that insulin accelerates the conversion of fat to sugar and that this gluconeogenesis protects the animal from hypoglycemia. In a reinvestigation Hawley found that after phlorhizination dogs are actually more sensitive to insulin than before phlorhizination. Neither the D:N ratio nor the respiratory quotient gave the slightest indication of a conversion of fat to carbohydrate.

Dann, Chambers & Lusk found that phlorhizin produces a smaller increase in O_2 consumption and less glycosuria in thyroidectomized than in normal dogs. This seems to be due to the fact that the usual rise in protein metabolism is absent. Interestingly enough, hypophysectomy (Biasotti & Houssay) has the same effect on phlorhizination as thyroidectomy, i.e., the capacity to form sugar at the expense of endogenous protein metabolism is diminished.

Wilson studied the rate of intestinal absorption of glucose, glycine, and alanine in phlorhizinized rats. Whereas glucose absorption was diminished, that of amino acids was increased by phlorhizination, so that all three substances were absorbed at about equal rates. This explains, according to Wilson, the equal rate of glucose elimination in the urine of phlorhizinized dogs after ingestion of these three substances.

EFFECTS OF HORMONES

a) *Pituitary*.—Bischoff & Long (1) found that pitressin (15 units per kilo) raised the blood lactic acid not only in normal but also in adrenalectomized animals, hence this effect is not a secondary reaction due to the liberation of epinephrine from the adrenals. In contrast to this it was shown that insulin failed to raise the blood lactic-acid level in adrenalectomized animals in spite of a severe hypoglycemia, while it had this effect in animals with intact adrenals, an observation which was also made by Cori & Cori. Pitressin produced sometimes a fall and sometimes a rise in blood sugar, it had little effect on glycogen stores, and it proved to be antagonistic to insulin hypoglycemia even in the adrenalectomized rabbit. Increases in blood lactic acid in unanesthetized dogs following the injection of pitressin were reported by Himwich, Haynes & Fazikas, while pitocin was

found to have little effect. Baumann & Marine and Houssay *et al.* produced hyperglycemia and glycosuria by injection of extracts of the anterior lobe, the former in rabbits and the latter in dogs and rats. Similar observations have been made by Evans (1931).

b) *Epinephrine and piqûre.*—In confirmation of results obtained by other workers, Major & Mann, (2), showed that epinephrine causes a decrease in muscle glycogen at rates of injection below those required to raise blood pressure. Brentano reports that the glycogenolytic effect of epinephrine can be demonstrated three days after cutting the motor nerve of muscle. According to Bischoff & Long, (2), total fermentable carbohydrate of skeletal muscle exceeds the sum of glycogen and fermentable muscle sugar by 100 to 300 mg. per cent, a difference which disappears after epinephrine administration. Eight hours after subcutaneous epinephrine injection, i.e., when the animals were recovering from the effects of the injection, Long & Bischoff observed a fall in fermentable muscle sugar in rabbits and rats. It is during this period that the blood sugar often falls to subnormal values and this would be reflected in the muscle-sugar values.

Effects produced by prolonged intravenous administration of epinephrine (4 to 13 days) in unanesthetized dogs are described by Samson & Jacobs. The elevated blood sugar returned to the initial value after 24 hours in spite of the continued injection. When the injection was stopped for a few hours after 48 hours, the blood sugar fell in each case to hypoglycemic values and then returned spontaneously to the normal level. Upon resumption of the epinephrine injection, hyperglycemia developed again and lasted for about 11 hours. The arterio-venous blood-sugar difference, which was small to start with, was not increased during the hyperglycemic phase. Nitrogen excretion was not changed by the injection.

Bell found that epinephrine and Kerr & Blish that insulin injections increase the hexosemonophosphate content of skeletal muscle, in confirmation of results obtained by others. Nitzescu & Munteanu showed that doses of ergotamine which suppressed epinephrine hyperglycemia in rabbits were without effect on the increase in blood lactic acid produced by epinephrine. Althausen & Thoenes described a test for carbohydrate tolerance which involves feeding of glucose followed by an injection of epinephrine. Ephedrine was found to resemble epinephrine in its effect on blood lactic acid and oxygen consumption of man (Coltrin).

Donhoffer & Macleod made decerebrations at different levels of the brain in order to localize the piqûre center of Claude Bernard. Transsections involving the hypothalamic region but still anterior to the pons, and transsections posterior to the pons, did not result in hyperglycemia. Transsections through the pons always resulted in hyperglycemia of several hours' duration. Other changes accompanying decerebration hyperglycemia were an increase in blood lactic acid and a decrease in muscle glycogen. Following adrenalectomy, decerebration through the pons produced little if any change in blood sugar, lactic acid, or muscle glycogen.

c) *Adrenal cortex*.—Britton & Silvette believe that the cortical hormone is primarily concerned in the maintenance of normal glucose and glycogen levels in the body. Following adrenalectomy there is a decrease in blood sugar, liver glycogen, and muscle glycogen, and these are restored to normal after treatment with cortical extract. An increase in blood sugar, liver glycogen, and muscle glycogen was also reported in normal fasted rats one hour after injection of cortical extract. Buell, Strauss & Andrus allowed muscle of normal, thyroxinized, and adrenalectomized animals to autolyse in NaCl solution. In the last two cases lactic acid was formed at a slower rate and came to a standstill sooner than in normal muscle. The myasthenic condition in adrenalectomized animals is ascribed to insufficient lactic-acid production for resynthesis of phosphocreatine.

d) *Insulin*.—The relation of insulin dosage to effect on blood sugar has been re-investigated by Scott & Dotti on very large groups of rabbits, so that the data could be treated statistically. The conclusions reached are that when the dose is measured in units per kilo and the blood is drawn 30 minutes after subcutaneous injection, the drop in blood sugar is proportional to the logarithm of the dose for amounts not less than $\frac{1}{16}$ and not more than $\frac{1}{2}$ unit of insulin per kilo of body weight. Contrary to previous statements the nature of the diet had no effect on blood-sugar changes after insulin.

The work of Quigley and co-workers and of Cleghorn & Peterson does not lend support to the conception that the vagus controls insulin secretion by the pancreas. The former workers found that dogs vagotomized one to six months before the experiment showed the same ability to metabolize glucose as normal dogs. The latter workers showed that when the leg muscles of decerebrate cats were fatigued by tetanization, glycogen reappeared after a resting period of $1\frac{1}{2}$ hours, even though the vagi had been cut. On the other

hand, Geiger claims that heating of carotid blood in chloralosed dogs produces hypoglycemia and that this effect is absent after the vagi have been cut.

A new effect of insulin has been described by Quick. It increases the excretion of glycuronic acid after administration of benzoic acid to dogs.

METABOLISM OF HEART

Macpherson, Essex & Mann found the glycogen content of the auricle higher than that of the ventricle. Glycogen disappeared at an increased rate from the perfused rabbit heart when the perfusion fluid contained no glucose. Long & Evans, experimenting on rats, observed a progressive increase in the glycogen content of the heart during fasting, while that of skeletal muscle diminished. Glucose alone had no effect, while injection of glucose plus insulin caused a marked increase in heart glycogen. Epinephrine injections or exercise, which lowered the glycogen content of skeletal muscle to 55 to 70 per cent of the control value, had little effect on heart glycogen. Breathing of 8 per cent CO_2 or ingestion of moderate amounts of bicarbonate had no effect on heart glycogen or muscle glycogen; bi-carbonate tetany caused a fall in both. Anoxemia led to a marked loss of heart glycogen, with prompt and complete recovery when the cause of anoxemia was removed. Clark, Gaddie & Stewart report that during perfusion of the well-oxygenated frog heart there appears a fermentable reducing substance which is formed from some unknown source and amounts to 0.4 mg. in a heart weighing 150 mg. Carbohydrate oxidation corresponded to 40 per cent of the total oxygen consumption.

GALACTOSE

May has described a new polysaccharide $(\text{C}_6 \cdot \text{H}_{10} \cdot \text{O}_5)_n$, which yields galactose upon acid hydrolysis, is not destroyed when heated in 40 per cent potash solution, has a specific rotation, $[\alpha]_D = -22.73$, does not give the iodine test for glycogen, and is not attacked by salivary diastase. The molecular weight is similar to that of gly-

cogen. This new polysaccharide, which is called galactogen, occurs in the snail, where it constitutes 17 per cent of the polysaccharides present, 83 per cent being present in the form of glycogen. The eggs of the snail contain only galactogen.

Roe & Schwartzman found no difference in galactose tolerance between normal men and women, as judged by galactose determinations in blood and urine. In diabetics the blood and urine galactose curve was essentially the same as in normal subjects. In rabbits insulin did not accelerate the disappearance of galactose from the blood. The utilization of galactose is considered to be independent of insulin and since diabetics have the same tolerance for this sugar as normal subjects, galactose is regarded as a valuable addition to the diabetic diet. Wierzuchowski (1931) has shown, however, that insulin increases the rate of utilization of galactose in dogs receiving a constant intravenous injection of this sugar.

Deuel, Gulick & Butts find that galactose possesses a greater ketolytic action than glucose in human ketosis due to fasting or in ketosis produced by a protein-fat diet. Galactose also has a more pronounced nitrogen-sparing action than glucose. Fructose and sucrose occupy an intermediate position between galactose and glucose in their ketolytic activity. In an attempt to explain these findings, Deuel, Mackay & Gulick tested the ability of rats to form liver glycogen from galactose. They observed an average value of 1.26 per cent after 6 hours of galactose absorption, which is in good agreement with a value of 1.16 per cent reported by Cori (1926) 4 hours after galactose feeding. Nine hours after galactose feeding the liver glycogen had risen to 1.88 per cent. The rate of glycogen formation in the liver from galactose was not compared with that of other sugars. Glucose and fructose form 4 to 5 per cent glycogen in 4 hours, and Deuel's data are therefore additional support for the contention that galactose is a poor glycogen-former in the liver of the rat when compared with glucose or fructose. Species differences have to be considered in investigations concerning galactose, the rat and dog having a particularly low tolerance for this sugar as shown by excretion of large amounts in the urine.

Carpenter & Lee determined the effects of 5 to 40 gm. of galactose on the respiratory exchange in a trained subject. The respiratory quotient rose even after 5 gm. and reached its peak between 15 and 30 minutes after ingestion. After larger amounts of galactose the peak of the quotient occurred at a later period, generally between

45 and 60 minutes. Seventy-five to 100 minutes after ingestion the respiratory quotient fell in all cases to a sub-basal level, an effect which was not seen after glucose or fructose ingestion. In a comparison of the respiratory exchange of men and women as affected by ingestion of galactose, women were found to exhibit a greater individual variation in all factors than men. When two sugars were ingested at the same time, glucose and fructose gave a simple summation of their separate effects, but galactose combined with either glucose or fructose caused a greater rise in heat production than would be expected from simple summation. Thus, 40 gm. of lactose gave the same rise in heat production as 20 gm. of galactose plus 20 gm. of glucose, i.e., about 8 calories, but the summated effect of glucose and galactose when these two sugars were ingested separately corresponded to an increase in heat production of only 1.5 calories.

A study of the value of galactose- and fructose-tolerance tests in patients with hepatic lesions has been made by Kimball.

LACTIC ACID

a) *Utilization.*—Hartmann & Senn determined the response of normal human subjects to intravenous injection of sodium *dl*-lactate (0.36 to 0.63 gm. per kilo). Fifteen minutes after injection about 90 per cent of the injected lactate had left the blood and there was a rise in true blood sugar of about 20 mg. per cent. The blood CO₂ reached its peak after 1 hour, while the most alkaline urine was excreted during the second hour. Under the assumption that the lactate was distributed through 67 per cent of the body weight a good agreement was obtained between expected and actually observed increase in blood CO₂. Dietrich & Zeynen measured the total extra oxygen consumption of normal human subjects following the intravenous injection of 4.8 to 16 gm. of sodium *dl*-lactate and found that an average of 20.9 per cent of the injected lactate was oxidized. In severe hepatic disease the disappearance of injected lactate from the blood was delayed, an observation which has also been made by Snell & Roth.

b) *Formation in muscle and other tissues.*—Macpherson studied the behavior of glycogen and lactic acid in haddock muscle after

death. He found that the glycogen disappeared rapidly during the first 3 hours. Lactic acid rose steadily but fell short of the glycogen, which disappeared in the 3-hour period. The fate of the glycogen which did not become lactic acid remained obscure. G. T. Cori kept muscle removed from amyatalized rats in nitrogen at 37° C. The disappearance of glycogen on the one hand and accumulation of lactic acid plus hexosemonophosphate on the other hand showed good agreement after 30, 60, and 120 minutes of anaerobiosis. Muscles of different species need not necessarily behave in exactly the same way after removal from the body. This is suggested by the fact that the glycolytic-enzyme system extracted from muscle of different species shows marked differences in its action.

Haldi, (1), studied the rate of lactic-acid formation in excised brain, kidney, muscle, testicle, and liver tissue under anaerobic conditions at body temperature for 10 to 20 minutes. The fastest lactic-acid formation occurred in brain, the slowest in testicular tissue. The lactic-acid curve in muscle and liver was a straight line; in other tissues lactic-acid formation fell off after 10 minutes. In dog muscle 5 mg. of lactic acid were formed per 100 gm. per minute, while G. T. Cori found a value of 3.6 mg. for rat muscle, the periods of observation being 12 and 60 minutes respectively. In Haldi's, (2), experiments intravenous injection of moniodoacetate produced complete inhibition of lactic-acid formation in kidney but usually not in brain tissue.

Haarmann compared the decrease in "total carbohydrate" (a separation into fermentable and non-fermentable reducing substances was not made) with the increase in lactic acid in minced muscle, kept anaerobically in phosphate buffer at pH 7.0. In rabbit muscle there disappeared on an average 389 mg. of total carbohydrate and there were formed 315 mg. of lactic acid. In dog muscle the difference was greater, the respective figures being 482 and 258 mg. Kerly & Ronzoni kept frog muscle in Ringer's solution of different pH under anaerobic conditions. At pH 9.0 the loss in total fermentable carbohydrate was approximately balanced by the gain in lactic acid, but at a neutral or acid reaction the lactic-acid increase fell short of the decrease in carbohydrate. At pH 6.0 no lactic-acid formation occurred for the first 3 hours. If anaerobiosis was continued for 20 hours, a large amount of a reducing substance fermenting at the same rate as glucose was formed.

In other papers Haarmann compared the amounts of lactic acid

formed by various minced tissues, kept anaerobically, from added glucose, fructose, hexosediphosphate, and glycogen. Whereas skeletal muscle formed large amounts of lactic acid from glycogen and hexosediphosphate and only small amounts from glucose, the reverse was the case with heart and brain tissue. In a later paper Haarmann & Stratmann claim, however, that if the glucose concentration is 0.1 instead of 0.2 per cent, skeletal muscle forms large amounts of lactic acid from added glucose. Addition of pyruvate led to lactic-acid formation in all tissues investigated. Addition of monobromoacetate in concentrations which completely inhibited lactic-acid formation caused anaerobic disappearance of considerable amounts of lactic acid. An interpretation of Haarmann's findings, many of which could not be reviewed here, seems impossible at the present time.

Dickens & Greville also studied the influence of addition of glucose and fructose on lactic-acid formation of various tissues, but they kept the tissues under more nearly physiological conditions, making use of the Warburg technique. Retina, submaxillary gland, spleen, brain, and nerve formed no lactic acid from fructose. Liver formed large amounts of lactic acid from fructose. Rat and fowl sarcoma showed a fructolysis amounting to $\frac{1}{3}$ of glycolysis. Other malignant animal tumors did not fructolyze. Sodium fluoride inhibited fructolysis in tumor and liver.

c) *Formation from methylglyoxal.*—A notable advance has been made in the elucidation of the mechanism of lactic-acid formation from methylglyoxal by animal tissues. It was shown by Lohmann that tissue extracts (liver or muscle from rats, rabbits, frogs) lose their ability to form lactic acid from synthetic methylglyoxal, when they are dialyzed or treated with oxygen in a solution of sodium bicarbonate. Addition of reduced glutathione brings back the ability to form lactic acid. Glutathione brings about the formation of more than 400 times its weight of lactic acid from methylglyoxal. Other substances which form complexes with heavy metals (HCN, cysteine, H₂S, pyrophosphate, citrate, oxyquinoline) cannot be substituted for glutathione. Amino acids, creatine, creatinine, adenylic acid, insulin, co-zymase, and oxidized glutathione were also found inactive. M/1000 copper or mercury salts completely inhibited the effect of M/5000 glutathione. Fe, Mn, Ni, Co, Zn, or Pb did not inhibit. When more glutathione was added, the inhibiting effect of Cu could be overcome. Glycogen can be glycolyzed by tissue extracts in the

absence of glutathione, i.e., it can form lactic acid under conditions under which methylglyoxal is unable to do so. Possibly methylglyoxal is not a normal intermediary of the glycogen-lactic-acid transformation. Another possibility is that synthetic and biological methylglyoxal are different, several tautomeric forms having been recognized; but after all methylglyoxalase extracted from tissues does act on synthetic methylglyoxal.

Washed, laked, and centrifuged ox corpuscles were found to convert added methylglyoxal (and *d*-, *l*-, or *dl*-glyceric aldehyde) to lactic acid (Embden & Metz). Curiously enough, the isolated zinc salts proved to be dextrorotatory in each case (*l*-lactic acid). This would also tend to cast doubt on methylglyoxal as an intermediary in blood glycolysis.

d) Formation in blood.—Meyerhof succeeded in separating the glycolytic enzyme from red-blood cells of various species (man, rabbit, ox, horse, goose, rat) by a method very similar to the one used for muscle. This enabled him to elucidate the mechanism of lactic-acid formation in erythrocytes and to compare it with that in muscle. When extracts made from rat erythrocytes were dialyzed, the glycolytic power was practically reduced to zero, but addition of adenosine-triphosphate plus magnesium returned it to its original level. The co-enzyme system in erythrocytes and muscle is, therefore, the same. As in muscle, an intermediary formation of hexosephosphate esters occurs. Addition of "hexokinase," an activator prepared from yeast, which was found to accelerate lactic-acid formation from glucose in extracts of mammalian muscle, had the same effect in extracts of rat erythrocytes. In contrast to muscle extract, erythrocyte extract is unable to glycolyze glycogen. Another, though minor, difference is that the hexosediphosphate which accumulates as an intermediary in both erythrocyte and muscle extracts is glycolyzed at a slower rate by the former than by the latter.

e) Pyruvic acid.—Case found that addition of starch or hexose-diphosphate to muscle extract gave rise to the formation of pyruvic acid, while methylglyoxal did not. Addition of antiglyoxalase increased the amount of pyruvic acid, and sodium fluoride completely inhibited it. Gorr & Wagner incubated minced-liver tissue with pyruvic acid and added calcium sulphite in order to bind any acet-aldehyde that might be formed. In the hands of previous investigators this "*Abfangverfahren*" had always yielded small amounts of acetaldehyde. Gorr & Wagner found that what appeared to be acet-

aldehyde did not give a typical Rimini test and they succeeded in isolating acetone from liver incubated with pyruvic acid. Their conclusion is that the liver contains no carboxylase. Wendel, (1), has continued his interesting observations on methylene blue catalysis of lactic-acid oxidation. It now appears that methemoglobin is not essential in the oxidation of lactate and the accelerating effect of HCN is due to a union with the product of oxidation, namely pyruvic acid.

METHODS

Harding and collaborators report the use of a yeast, acclimatized to galactose, for the determination of this sugar in blood and urine, and they have elaborated a system of analysis for mixtures of mono-saccharides based on the selective action of various micro-organisms. Vaughan & Hubbard found that *B. coli* grown on a sugar-free broth destroy large amounts of glucose and mannose, less of fructose, galactose, and maltose, and none of lactose, xylose, and arabinose. When grown on a medium containing the last-named sugars, the bacteria acquire the power to destroy them.

Two procedures for the measurement of diastatic activity have been described by Somogyi. Raw starches of different origin as well as glycogen were found to give identical results when used as substrates, whereas soluble starch gave different results for each batch. The limits of enzyme-substrate ratios were established within which the quantity of reducing sugar produced was directly proportional to the amount of enzyme present.

Steiner, Urban & West introduced new and efficient ways of precipitating biological fluids (whole blood, plasma, spinal fluid, milk) by means of iron and thorium salts followed by treatment with barium carbonate. True sugar values are obtained in these filtrates when the Shaffer-Hartmann method is used. Wendel described a method for the determination of pyruvic acid, which is based on its reduction to lactic acid by means of the Zn-Cu couple in sulfuric acid. Freshly re-distilled pyruvic acid, in contrast to old and polymerized material, could be reduced completely and was recovered quantitatively when added to blood precipitated with mercuric chloride. Certain improvements were also introduced in the Friedemann-Cotonio-Shaffer method for lactic acid.

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FAT METABOLISM*

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Severe limitations on the amount of space available have necessitated the omission of many papers, including a number devoted to analytical methods, cholesterol, effect of vitamins on absorption, lipase, and esterase.

LIPID ABSORPTION

Rôle of the pancreas in the absorption of fat.—The question of the effect of the pancreas on the absorption of fatty acids is dealt with by Nothmann & Wendt (1) who find that the absorption of the fatty acids, unlike the digestion of neutral fat, is independent of pancreatic secretion.

Utilizability of caprin.—Powell (2) examined the utilizability of tricaprín and found that it could be stored to the extent of about 15 per cent of the depot fat. Earlier work by herself (3) and others demonstrated that butyric and caproic acids are not stored, caprylic acid only in traces, lauric acid to as high as 25 per cent, and myristic acid to 17.5 per cent. Chains of eight or fewer carbon atoms tend to disappear or to be changed to longer chains.

Absorption of cholesterol and coprosterol.—The absorption of the sterols has been investigated by Bürger & Winterseel (4), who comment on the fact that the sterols are largely eliminated through the large intestine, while cholesterol is largely absorbed in the upper intestine. Since, therefore, absorption proceeds in one place and excretion in another, they looked for a change in the sterol content of the intestine. They fed cholesterol and coprosterol in olive oil with olive oil as a control. Coprosterol was not absorbed, nor was dihydrocholesterol, while cholesterol was readily absorbed and a considerable proportion of it remained in the body after six days. They conclude that hydrogenated sterols are not absorbable.

Absorption of ergosterol.—Schönheimer and co-workers (5) found that laying hens absorbed a small but significant amount of ergosterol while they had previously (6) shown that unirradiated

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ergosterol is not absorbed by mice, rats, rabbits, or dogs. Menschick & Page (7), on feeding ergosterol to hens, found a 40 to 50 per cent increase in the ergosterol content of eggs, starting the fourth day after the feeding was begun and ending at about the same time after it was discontinued.

Absorption of phytosterol.—Nikuni (8), experimenting with mice, found both cholesterol and phytosterol were absorbed, stored, and partly excreted. Phytosterol is apparently absorbed as well as cholesterol but is not stored to as great an extent.

Solubility of cholesterol in bile salts.—Spanner & Bauman (9), experimenting on the solubility of cholesterol in bile salts, found that, although cholesterol is insoluble in water, it may be soluble to the extent of 1 per cent in bile and that normal bile is often not saturated with cholesterol but will dissolve more.

LIPIDS OF THE BLOOD

A. NORMAL

Childhood.—Ward (10) found that the cholesterol content of the blood of normal boys, 6 to 13 years old, increases with age and shows but slight individual variations. The values for normal girls of the same age show greater variations.

Irradiation.—Leopold, Bernhard & Tow (11) found that irradiation with ultra-violet light increased the blood lipids in one-quarter of the cases studied. Inunctions with the irradiated wool fat increased the cholesterol, as did also inunctions with plain wool fat, followed by irradiation in most of the cases with low cholesterol, and in one-third of the normals.

Blood coagulation.—Barbieri (12) found that cephalin extracted from various organs accelerates the coagulation of recalcified oxalated plasma. Splenic cephalin appears to be the more active. The increase in the coagulability of the blood after bleeding is due to an increase in cephalin.

Lipid-protein combinations in blood.—Macheboeuf & Sandor (13) report that ether alone, even in several days, will not extract a notable amount of lipids from blood serum. If, however, 5 to 13 per cent of alcohol is added to the serum and the mixture allowed to digest for a while, about 50 per cent of the lipids are extractable by ether. The proteins do not appear to be affected except that the globulins, when separated, are free from lipid. The lipids attached to the albumin resist extraction under these conditions.

Injection of phospholipid.—Pasternak & Page (14) injected rabbits with emulsions of cephalin. After massive injections the lipid content of the plasma is increased for a long period. Half an hour after a large cephalin injection there is a strong increase in the phosphatide content of the liver, although the fat content remains unchanged at first. Soon, however, this also increases and remains high when the phosphatide content has fallen to its initial level. No change was observed in the phosphatide content of the brain. Injections of small doses of cephalin over a long period of time leave the plasma phosphatide content normal, although the total quantity of cephalin injected may be great. An increased phosphorus excretion in the urine was found occasionally.

Absorption of fat.—The effect of absorbed fat on the blood lipids in the lower animals has been very well shown a number of times. In the case of human beings, the feeding of fat meals has sometimes been found to result in an increase in the blood lipids and sometimes not. The question has been reinvestigated by Man & Gildea (15) at Yale. They found that when a sufficiently large dose of fat was given to normal men and women, for example, $3\frac{1}{2}$ to 4 grams per kilo of body weight, there was always a marked rise in the serum fatty acids and a moderate increase in serum phospholipids. Also the ingestion of a balanced meal, containing as little as $\frac{1}{6}$ of a gram of fat per kilo and with carbohydrate plus protein slightly exceeding the weight of the fat, produced a rise in serum fatty acids in normal men and women. This latter finding is contrary to the results obtained by earlier workers, for example, McClure & Huntsinger (16) and Bang (17). [See also *Ann. Rev. Biochem.*, 1, 274 (1932).]

Nissen (18) gave to a number of normal resting persons doses of 1 gram of fat per kilo and noted the resulting lipemia curve. It rose slowly, reaching a maximum value in 4 hours, then dropped off rapidly, returning to its initial level in 6 to 7 hours. In similar persons not at rest the curve rose more rapidly and the maximum value was obtained in 3 hours, although it was somewhat less than in the resting individuals. In the fasting state the lipid content of the venous and capillary blood was the same, but during alimentary lipemia it was distinctly lower in the venous blood when the curve was at its maximum. The alimentary lipemia reaction in children is smaller than in the adult, the curve attaining its peak in 2 to 3 hours and the maximum values being less.

Diet.—The effect of diet on blood lipids in dogs was studied by

Ling (19) over a period of six months. The blood fat decreased on a fat-free diet and dropped further on starvation. On the fat-free diet the decrease was due largely to lipids other than lecithin and cholesterol. When olive oil was fed, the values for all the lipids except free cholesterol rose. Since the cholesterol ester fraction nearly doubled, although no cholesterol was present in the oil given, it is concluded that cholesterol must have been mobilized for the purpose of transporting about one-quarter of the ingested fatty acids.

Lactation.—LeRoy, Lecoq, Veline, Valissant & Barjot (20) report that there exists a high coefficient of correlation between the total fatty acids in the blood and the butter-fat content of the milk. It is suggested that the determination of fatty acids in the blood will furnish a value by which the future capacity of young cattle to produce butter may be established.

Schaible (21) found that the lactation values for total fatty acids and phospholipids were double those in the non-lactating state in the blood of cattle, while the nature of the total fatty acids was much the same in both. The yield of phospholipid fatty acids was always much below the theoretical value. The fatty acids in combination with cholesterol gave an iodine number too low for linolic acid, indicating therefore an admixture with oleic acid, but the values were higher than those of the fatty acids of the food so that there must have been a selection of the more unsaturated acids from the mixtures presented in the food. The amount of unsaturated acids combined with cholesterol is greater than that combined in lecithin in the lactating blood. His conclusion is that cholesterol aids in the transport of the unsaturated fatty acids.

Diurnal variations.—Bruger & Somach (22) review recent work on this topic, mentioning that of Glusker (23), McClure & Huntsinger (16), and McEachern & Gilmour (24). Glusker (23) found in dogs over an 8-hour period, post-absorptive, a standard deviation of ± 3.7 per cent from an average value. McClure & Huntsinger (16) found the cholesterol to be constant in the 7-hour daily period in young adult humans. McEachern & Gilmour (24), however, found large variations in the plasma cholesterol of humans under the same conditions and by the same methods. The work of Bruger & Somach (22) agrees with that of Glusker and of McClure & Huntsinger and does not support that of McEachern & Gilmour. They determined whole-blood cholesterol every 2 hours for 24 hours under normal conditions of feeding and found a standard deviation of ± 8

per cent for the whole period and ± 3.5 per cent for the morning hours. A fasting group of 9 individuals from whom samples were taken every hour for 4 hours gave results with a standard deviation of ± 3.9 per cent. Ingestion of food had no appreciable effect. They suggest that the differences between these various workers may have been due to technique since Mirsky & Bruger (25) found temperature control during the development of the color very necessary for accurate work.

B. DISEASE

Epilepsy.—Gray & McGee (26) found that the average cholesterol value of whole blood from men was 194 mg. per 100 cc. in normal persons, 165 mg. in epileptics, and 154 mg. in feeble-minded persons. McQuarrie, Husted, Bloor & Patterson (27) made a statistical study in 100 epileptics and 100 normals. No significant difference in the range of values of cholesterol or its variability in the two groups was found. The mean value for lecithin was significantly lower and the total fatty acids higher in epileptics than in normals and these two appear significantly more variable in epileptics than in normals. McQuarrie, Husted & Bloor (28) made studies of the lipids in the blood of epileptics with reference to the periods of seizure. The levels of the various lipids, while at the low end of normal, could scarcely be classed as abnormal but the lecithin/cholesterol ratios were consistently higher near the time of seizure. In the same individual the ratios were highest in those samples nearest the seizure and lowest in those remote from a seizure. Lecithin tended to increase absolutely as well as relatively at or near the seizure in some instances. Cholesterol changes were less apparent but when they did occur they tended to go in the opposite direction. While lecithin is a hydrophilic colloid, cholesterol is a hydrophobic colloid. The possible effects of these substances on the water balance in the controlling tissues in these cases are discussed and compared with a similar discussion by Haustein (29), who found that the cholesterol content of the blood of eight children with disturbed water metabolism was considerably below a normal level.

Anemia.—Chamberlain & Corlett (30) produced anemia in rabbits by bleeding and by a subcutaneous injection of phenyl hydrazine hydrochloride. In whatever way the anemia was produced variations in the cholesterol content of the blood and plasma took the same form, namely, an initial fall followed by a secondary rise. This indi-

cates that the hypercholesterolemia in anemias is not peculiar to the anemia of hemorrhage. The reason for the hypercholesterolemia is not obvious but as cholesterol possesses notable antihemolytic properties, it may be a protective phenomenon to prevent any further destruction of corpuscles.

Hypertension.—Wacker & Fahrig (31) found the total serum cholesterol high in 75 per cent of their patients, averaging 200 mg. per 100 cc. as compared with a normal average of 152 mg. per 100 cc. The increased cholesterol is only incidental to a general serum hyperlipidemia, involving phosphatides, cholesterol esters, neutral fats, and free cholesterol, all of which show a roughly parallel increase.

Medvei (32) found that, although patients with arterial hypertension have on the average a somewhat higher blood cholesterol than normal, the difference is not significant. Contrary to the findings of Alvarez & Neuschlosz (33) the blood serum of hypertensive patients is not supersaturated with cholesterol: only one of 15 sera proved to be supersaturated, and all sera, of normal and hypertensive patients alike, were found to be saturated.

Liver disease.—Epstein (34) reviews this topic, noting that obstructive jaundice has long been known to increase the blood cholesterol, while in other than obstructive jaundice, cirrhosis of liver, acute yellow atrophy, pernicious vomiting, the cholesterolemia bore no relation to the jaundice. In conditions of liver damage he found that the blood cholesterol-ester content was low. Jaundice due to stoppage always produced hypercholesterolemia while in biliary diseases without obstruction there was little change. In parenchymatous diseases of the liver there is a drop in ester in the blood parallel to the parenchyma damage.

Stroebe (35) found that the blood cholesterol is variable in hepatic cirrhosis, but with the beginning of jaundice of hepatic origin the cholesterol ester decreases, ranging from 13 to 43 mg. per 100 cc. as compared with normal values of 56 to 80 mg. per 100 cc. A fall in the cholesterol ester is good evidence of the progress of hepatic disease. Thus there seems to be agreement that a properly functioning liver is necessary for the formation of cholesterol esters in the blood plasma.

Infections.—McQuarrie & Stoesser (36) have made a study of the influence of acute infections and artificial fever on plasma lipids. Their results may be briefly summarized as follows: In every case of acute infection studied, there was at the height of the disease a

definite lowering of cholesterol, lecithin, and total fatty acid. In all but one case the values became definitely subnormal but rose again to normal during convalescence. There appears to have been no constant relationship between the height of the fever and the degree of hypocholesterolemia or hypolecithinemia in these cases. Diet had no demonstrable influence on the results. The total leucocyte count tended to vary inversely with the lipoid content of the plasma. That elevated body temperature *per se* is not responsible for the lowering of the various lipoid constituents is indicated by the fact that fever, artificially produced by phenylethylhydantoin, failed to have any such effect. These results are in agreement with those noted in the review of this topic last year,¹ and seem to leave little doubt that changes in the blood lipids, not only in cholesterol but all the lipids, are brought about by infections.

INTERMEDIARY METABOLISM

The liver in lipid metabolism.—Rosenfeld (37) found that the essential difference between the fatty liver of phosphorus- and phlorizin-poisoning is that the fatty liver of phlorizin poisoning is prevented by glycogen formation if sugar is given along with the drug, while in the case of phosphorus, no feeding will prevent the fatty liver because no glycogen is formed. Sugar is effective in the phlorizinated animal only when given by mouth and not intravenously. Adrenalin prevents the fatty liver of phosphorus poisoning. Feeding fat to dogs in amounts of 30 grams per kilo gives a fatty liver in four days but it is prevented if 7 to 8 grams per kilo of sugar are fed at the same time. Experimental fatty kidneys can be produced only by fat feeding and with great difficulty.

The degree of fat infiltration in the liver in starved rats was found by Dible (38) to be dependent on the quantity of fat available for mobilization to the depots.

Best, Hershey & Huntsman (39) report that when white rats of 150 to 250 grams weight are fed a suitable diet for 3 weeks their livers contain 15 to 18 per cent fatty acids with an iodine number of 100. Comparable groups fed the same diet plus lecithin have only about 5 per cent of fatty acids in their liver with an iodine number of about 132.

Later work by Best & Huntsman (40) showed that choline is the

¹ *Ann. Rev. Biochem.*, 1, 291 (1932).

only constituent of lecithin which inhibits the deposition of fat in the livers of normal rats on a high-fat diet. Betaine seems to have an effect similar to that of choline.

Acute changes in the fat content of liver have been produced by Hynd & Rotter (41) by the subcutaneous injection of pituitrin and pitressin, either of which produced in carbohydrate-fed rats an increase of the fat of the liver accompanied by a decrease of the carbohydrate during the first 5 hours. Later there was a reversal of these changes. Muscle glycogen rises during the first 5 hours to about twice the normal level at which it is maintained for 24 hours.

The migration of the fat to the liver is claimed by Wertheimer (42) to be under nervous control because the fatty liver produced by phlorizin in dogs can be prevented by section of the spinal cord at the level of the sixth thoracic vertebra.

Infections with diphtheria were found by Scheff & Horner (43) to cause a disappearance of fat from the liver, while paratyphoid infection had no effect. Trypanosome infections produced a fatty liver. These changes are in the fat alone. Phospholipids and sterols are unaffected even when the fat accumulations are large or when the fat entirely disappears. The lecithin/cholesterol ratio remains constant. The conclusion is that phospholipid has no part in fat metabolism in the liver.

Fatty acid metabolism.—Contrary to the theory of Rosenfeld that "fats burn in the fire of carbohydrates," Rosenthal (44) found that fasting mice, with an extremely low carbohydrate content, oxidize β -hydroxybutyric acid as well as normal animals. Feeding β -hydroxybutyric acid to such animals causes no increase in liver glycogen, indicating that fatty acids are not converted to carbohydrate.

Magistris (45) has prepared from beef hypophyses (anterior lobe) a hormone which, when injected into rabbits, brings about a rise in the acetone bodies of the blood, the maximum rise occurring in 2 to 3 hours. The β -hydroxybutyric acid is especially increased.

That β -oxidation is not the only method of oxidation of the fatty acids is evident from the work of Raper & Wayne (46) and latterly of Quick (47), who discusses the topic with a summary of evidence. In his own work he finds that phenylisocrotinic acid is burned just as well as phenylbutyric acid, indicating that the organism can shift double bonds and that oxidation does not necessarily occur at the double bonds.

Witzemann (48) finds that in the oxidation of the α -hydroxy

fatty acids either one or two carbon atoms may be split off, depending on the length of the chain. In the three-carbon acid (lactic) one carbon is lost. In the higher acids two carbons are lost, α -hydroxybutyric acid being the pivot acid, sometimes losing one and sometimes two carbon atoms. Therefore, what is ordinarily regarded as β -oxidation may follow α -oxidation. Excess of alkali favors the loss of two-carbon fragments, one carbon being lost when the compound is in the keto state, two when it is in the enol state.

Fat to carbohydrate.—The change of fat to carbohydrate has been studied by Hawley (49) who examined the respiratory quotients and the urinary D/N ratios of phlorizinized fat dogs with and without insulin injections. No evidence of conversion of fat to carbohydrate was found.

Chambers and co-workers (50) repeated the work of Chaikoff & Macleod (51), who found an increase in the respiratory quotient in exercised depancreatized dogs and thought that it indicated the ability of the animal to burn sugar. The authors' experiments confirmed the rise in respiratory quotient but they believe it is due to changes in the carbon-dioxide equilibrium in the body following the rise in blood lactic acid which is also present. After the period of exercise the respiratory quotient fell below the resting level and the blood lactic acid returned to normal. They favor the theory that fat is the fuel of exercise in the depancreatized dog.

Rony, Mortimer & Ivy (52) offer some indirect evidence regarding the transformation of fat to sugar. They quote the work of Hoffmann & Wertheimer (53) and of Schur & Löw (54), who claim that the reserve fat in fasting animals is not conveyed as such but is transformed in the fat depots into glycogen and sugar, the latter being the material conveyed by the blood. Rony, Mortimer & Ivy find that the sugar content of the lymph of fasting and phlorizinized dogs is always less than that of the blood or of normal lymph, which they think speaks strongly against the transport of mobilized fat in the form of sugar.

Fat-deficiency disease.—Burr, Burr & Miller (55) found that both linolenic and linoleic acids are effective in curing rats suffering from a fat deficiency. They seem to be of about equal value, while oleic acid is ineffective; and α -eleostearic acid, an isomer of linolenic acid, is also ineffective. Methyl arachidonate has a slight depressing effect.

Evans & Lepkovsky (56) confirm the fact that a specific deficiency disease is produced on rigidly fat-free diets and is cured by

the administration of certain unsaturated fatty acids. Vitamin B has been ruled out. Cornstarch and rice starch are effective in curing deficiency, while potato starch is ineffective. These curative carbohydrates owe their effectiveness to their slight but important content of unsaturated fatty acids, presumably linoleic acid. Hog-liver glycogen is ineffective.

The necessity of linoleic or linolenic acids in the diet of growing rats is confirmed by Tange (57). He confirmed the finding that oleic acid was not adequate. Rossi (58) interjects a small factor of doubt into the unanimity in this field. He finds that rats receiving with their diet an amount of lipids equal to less than 2 per cent of the total calories, and receiving also large amounts of vitamin B and lipid-soluble vitamins, developed equally as well as the control rats which received the usual quantity of lipids. If lipids are necessary the amount must be very small.

SECRECTIONS AND EXCRETIONS

Rony, Mortimer & Ivy (52) find that the lymph of fasting or phlorizinized dogs contains more fats but less sugar and cholesterol than the blood drawn at the same time. After 2 to 14 days of fasting the total fatty acids of the lymph vary from 250 to 1,030 mg. per 100 cc., while the value for the blood is 157 to 371 mg. per 100 cc. The lymph fat is therefore derived principally from the fat stores and not from the blood. These findings raise the possibility that the lymph may be an important carrier of fat from the stores as well as from the intestine via the thoracic duct.

The composition of the crop milk of pigeons has been examined by Reed, Mendel & Vickery (59). They find that it contains: solids, 15 per cent; fat, 10 to 12 per cent; while the iodine number of the cold-alcohol extract is 106, of the hot extract 115. The milk also contains adequate amounts of A and B vitamins, which explains the very rapid growth of the young pigeons.

A comparison of the composition of exudates and transudates was made by Macheboeuf & Fethke (60). There is very little difference in the composition of the two classes of fluid, although in general the transudates are much lower in protein and lipids.

The lipid content of intestinal mucosa has been determined by Sperry (61). The fatty acid in the colon mucosa was less in amount than that in the mucosa of the small intestine. The average endogenous lipid excretion was 219 ± 9.5 mg. per kilo of weight per

week for normal dogs, and 615 ± 25.2 mg. for bile-fistula dogs. Sperry & Angevine (62) determined the lipid excretion in loops of the lower ileum and cecum and by ligation in the colon. Large secretions of lipid into the small intestine and small secretions into the colon were found. The high sterol content of the lipids indicated that they were not of bacterial origin. Since the amounts secreted were much greater than had been found in the feces, some degree of reabsorption by the large intestine is indicated.

Hill & Kohler (63) found that on a low-fat diet the administration of epinephrin in subglycosuric doses usually caused a definite rise (100 per cent) in fecal lipid excretion on the day following.

LIPIDS IN ANIMAL TISSUES

A. NORMAL

Brain.—Bülow & Page (64) found that freshly prepared cephalin from brain has values of carbon, hydrogen, nitrogen, and phosphorus which are too low, and the deficiency becomes greater as the material stands in air, owing to oxidation.

Page & Rudy (65) report a brain cephalin which yields a fatty acid percentage of 75, which is close to the theoretical content and much higher than has been reported previously for any cephalin. In brain cephalin they found stearic acid 30 per cent and a C₂₂ unsaturated acid 22 per cent, which was apparently a mixture of 4 and possibly 5 double-bond acids but contained no triple-bond acids. Unsaturated acids of the C₁₈ series amounted to 22 per cent.

Yokoyama & Suzuki (66) obtained, on hydrolysis of lecithin from 10 human brains, oleic, arachidonic, and palmitic acids from the α -lecithins and oleic, linolic, arachidonic, and palmitic acids from the β -lecithins.

Ernst Schmitz (67) found that the percentage of water in the cerebral hemispheres of rabbits was 76.73 to 81.10, and in cats 74 to 79.1 per cent. The phosphatide content was 4.9 to 5.0 per cent of the fresh substances in guinea pigs, 4.05 to 5.45 in rabbits, and 4.38 to 5.32 in cats. The cholesterol content was 1.45 to 1.55 per cent, 1.56 to 1.85 per cent, and 1.61 to 2.62 per cent, respectively. The cerebroside content was 0.63 to 0.88 per cent for rabbits, and 1.20 to 1.32 for cats. Very little difference was revealed in the composition of the two hemispheres.

The unsaturated acids of the ether-soluble phospholipids of brain were examined by Klenk (68) who found that the C₁₈ members con-

sisted almost entirely of oleic acid; that the C_{20} group averaged 3.1 double bonds and consisted of $C_{20}H_{38}O_2$ with a melting-point of 20° C., and arachidonic acid, $C_{20}H_{32}O_2$. The C_{22} acids averaged 4.4 double bonds and consisted of clupanodonic acid ($C_{22}H_{34}O_2$), and some unknown 5-bond acids. It is significant that the unsaturated C_{20} and C_{22} acids, so characteristic of fish-liver oils, occur also in land mammals, especially as regular constituents of phosphatides of the lecithin and cephalin type.

Skin.—Factors controlling the proportion of cholesterol in human skin were examined by Roffo (69), who found that the proportion of cholesterol in human skin is increased by exposure to sunlight. The skin from various parts of the face contains 2 to 7 times as much as the skin from the abdomen of the same person. The distribution in the face is roughly proportional to the extent of exposure to the sun. He found a direct relationship between frequency of skin tumors and the proportion of cholesterol in the skin. The higher the cholesterol the more frequent the occurrence of tumors.

In adults there is much more cholesterol in the skin of the face than in the skin of the back (70), but this is not true in the case of the fetus or the infant. In the infant the cholesterol content of the skin of the face increases with age. The differences are explained on the basis of exposure to sunlight.

Bone marrow.—Bone marrow was examined for its lipid content by Cheng (71). The gelatinous marrow contained 20 to 22 per cent more unsaturated acid than the yellow marrow. The saturated acids were mainly palmitic and stearic acids. The unsaturated acids were mainly oleic with some arachidonic. The phospholipid content was higher in the marrow of the calf than in the adult animal but the nitrogen content was the reverse.

Human fat.—The composition and distribution of the fat in the human subject have been investigated by Cathcart & Cuthbertson (72) in 14 subjects, ranging from 18 to 57 years of age. Cholesterol and lecithin were removed before analysis and corrections were made for the presence of cholesterol esters and for nitrogen and phosphorus compounds which had not been removed. For details on these data the reader is referred to the original article.

Distribution of animal fat.—Some of the distribution factors of fat were studied by Reed, Anderson & Mendel (73), using rats fed on a ration rich in cocoanut oil. Some were completely ovariectomized and others were given thyroxine. It was found that if the

ovaries were removed between the ages of 3 and 4 weeks the percentage of fat in the body was practically the same as that in control animals after 7 to 14 weeks and the iodine number of the subcutaneous fat was not altered. The ovariectomized animals, however, stored less fat in the genital tissues and more in the subcutaneous layers. When thyroxine was administered the percentage of depot fat in the entire body was less than half that found in the controls, but the distribution in the depots in both cases was similar. The fat produced by the animals fed thyroxine was more unsaturated. Summing up, they say, "of all the factors including food, under-nutrition, fasting, muscular activity, ovariotomy and the administration of thyroxine, which have so far been studied, the character of the diet and the thyroid hormone represent the only influences that have appreciably altered the quality of depot fat."

Liver.—Fränkel and co-workers (74 and 75) have isolated lignoceryl-sphingosine from liver. The yield was about 0.06 per cent of the dry weight. The fatty acids of liver phospholipids and fat have been examined by Klenk & Schönebeck (76). The fatty acids of the liver phospholipids were found to be the same as those of the brain, but in the brain the C_{22} acids predominated, while in the liver the main unsaturated acids were C_{20} . In the brain only traces of C_{18} acids of a higher degree of unsaturation than oleic acid were found, but in the liver linolic acid is abundant. The depot fat of beef contains nothing higher than C_{18} acids, while the neutral fat of liver was found to contain C_{20} and C_{24} , highly unsaturated acids which were not represented in the depot fat. They note that in general, wherever there are notable amounts of unsaturated acids, they are mostly C_{18} , C_{20} , and C_{22} . Acids of 18 carbon atoms are less important in animals, while in plants the highly unsaturated acids are mostly of this series.

B. ABNORMAL

Schüller-Christian disease.—Chemical analyses of the lipids of organs in a case of Schüller-Christian disease are given by Kleinmann (77). In the spleen the cholesterol/lecithin ratio was in marked contrast to that recorded in the Niemann-Pick disease (2.7 : 1 as compared with 1 : 9.3), as well as to that observed in normal spleens. Cholesterol esters greatly surpassed free cholesterol. Similar relations to those noted for the spleen were found in the dura, liver, and subcutaneous fatty tissue.

Yellow fever.—A study of the lipids of tissues in yellow fever in monkeys has been made by Gerard, Moissonnier & Welti (78), and they found that the disease causes a lowering of the lipoïd phosphorus, and an important diminution in the unsaponifiable fraction, which could not be explained by the lowering of cholesterol and the invariability of the constant factor in the lipid content of tissues.

Cancer.—The importance of a study of the cell lipids in cancerous tissues has been shown by Yasuda & Bloor (79), Bierich, Detzel & Lang (80), and LeMay (81), all of whom agree that malignant tumors have a much higher content of phospholipid and cholesterol, especially phospholipid, than do benign tumors or tissues on which the tumors are growing.

Bolaffi (82) finds sulphur-containing phospholipids in both human tumors and rat tumors. The sulphur and phosphorus contents vary with the different classes of tumors. A possible relation between cholesterol content and frequency of tumors has been noted in skin by Roffo (70). The higher the cholesterol content the greater the liability to tumors.

The lipid of blood in cancer cases has been examined by several workers (83, 84, 85, 86), with the idea of showing some relation between the lipid content of the blood and the cancerous condition, but without positive results.

Lecithin/cholesterol balance.—A review of the available literature on this topic has been made by Degkwitz (87). While there is much that is indefinite and contradictory in the data available, the results as a whole leave little doubt as regards the essential antagonism of these two substances in cellular physiology, and the importance of a study of the balance of the two in many important physiological phenomena is indicated.

Price & Lewis (88) found that the maximum value of surface tension of lecithin is at a pH of 2.6, which is close to the isoelectric point (2.7). Cholesterol had no effect on the surface tension of water. Lecithin solutions are turbid with units of radius 10^{-4} or 10^{-5} cm. and these are the capillary active particles. The isoelectric point of lecithin is shifted by cholesterol to pH 4. Determinations of surface tension were made by the Sugden maximum-bubble-pressure method.

That cholesterol aids the diffusion of acids and alkalies into gelatin and agar jellies, while lecithin slows it, was found by Magistris (89). In high concentration both slow the diffusion. The effect of lecithin is stronger.

The influence of phosphatides on proteolytic enzymes was studied by Rondoni (90). Two synthetic phosphatides, distearinlecithin and distearincephalin, both of which form stable emulsions, were tested for their influence on the activity of purified proteolytic enzymes. Lecithin inhibits proteinase and carboxypolypeptidase before, but not after, activation by enterokinase. Cephalin inhibits proteinase but has no effect on carboxypolypeptidase. Both phosphatides inhibit dipeptidase but are without influence on aminopolypeptidase.

LIPIDS OF BACTERIA AND MOLDS

Chargaff (91) found a striking difference between the diphtheria bacteria and the acid-fast tubercle bacteria, studied by Anderson and co-workers, in their low wax content and also their low total-lipid content. Chargaff (92) found that acid-fast non-pathogenic bacteria, as represented by turtle and smegma bacteria, differ from the related human and bovine pathogenic tubercle bacteria in their lower wax and higher fat content. The fat, like that of the tubercle bacteria examined by Anderson, consists partly of true glyceride, and partly of a fatty acid compound with a polysaccharide. The unsaponifiable portion gave none of the usual reactions of the sterols.

Uyei & Anderson (93), comparing the lipoids of the closely related organisms *B. leprae* and the tubercle bacillus, found that the most notable difference is in the lower phosphatide and wax content of the former. Pangborn, Chargaff & Anderson (94) reported on the "fat" of the timothy bacillus and found that it was not a glyceride but a compound of a carbohydrate or a polyhydric alcohol. The unsaponifiable matter was a dark oil with an iodine number of 126, which gave no sterol reactions. The solid saturated fatty acids consisted mainly of palmitic acid. The liquid acids, with an iodine number of 65, consisted mainly of a liquid saturated acid, probably tuberculostearic.

LIPIDS OF PLANTS

Hée & Bayle (95) in a study of the germination of the seeds of *Lupinus albus*, in light and darkness, found that the lecithins of the seed are largely used up for the development of the young plant, either as constructive elements or as combustibles, which, however, does not exclude their possible rôle as bioplastic agents as Zlataroff (96) has assumed.

Smith & Chibnall (97), studying the lipids of cocksfoot grass

(*Dactylis glomerata*), found lecithin, cephalin, and calcium phosphatidate. The fatty acids present were linolic, linolenic, and saturated acids, but no oleic acid. The ratio of saturated to unsaturated acids was less than unity, as is usual in plant phosphatides. In earlier work Channon & Chibnall (98) had found in cabbage leaves that lecithin and cephalin were absent and the main constituent was calcium phosphatidate.

Collison & Smedley-Maclean (99) found that the unsaponifiable matter of green leaves contained carotene, a sterol ($C_{27}H_{46}O$), ceryl alcohol ($C_{27}H_{54}O$), and the hydrocarbon, $C_{31}H_{64}$.

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THE METABOLISM OF PROTEINS AND AMINO ACIDS*

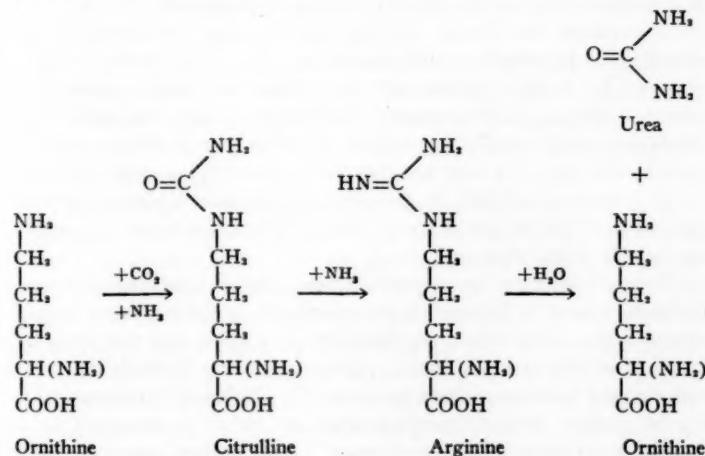
By J. MURRAY LUCK

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Owing to the exigencies of space it is possible to consider but a fraction of the many papers which deserve attention. A number of topics have had to be excluded in entirety for the present, while those accepted for discussion are necessarily incomplete. The current literature on protein storage, endogenous protein metabolism, arginase, certain individual amino acids, and amino-acid toxicity will be considered in a later volume.

UREA

Formation of urea.—One of the most substantial contributions to this subject has been furnished by Krebs & Henseleit (1). By use of the organ-slice method of Warburg, slightly modified, the authors have succeeded in showing that urea formation in the animal body probably proceeds as follows:



* Received February 15, 1933.

The evidence and conclusions may be summarized: (a) Of 14 amino acids and 5 nitrogenous bases investigated, only ornithine markedly increased the rate of formation of urea by liver slices suspended in a lactate-ammonia-inorganic-salt medium. (b) In later experiments, citrulline was found to be as effective as ornithine in promoting urea formation. The ratio, "ammonia disappeared"/"urea formed," in the presence of ornithine, was 1.81 (theoretical value 2.0) and in the presence of citrulline 1.38 (theoretical value 1.0+). Therefore citrulline is an intermediate. (c) In one experiment 30.4 mols of extra urea per mol of ornithine were formed. The ammonia content of the medium decreased, but the amino-nitrogen content remained unchanged; in the absence of added ammonia, urea was not formed. Therefore ornithine functions catalytically. (d) Urea formation is restricted to the liver. Sixteen other organs gave negative results. (e) The first reaction is considered to be slow, and the second is rapid. Both reactions depend upon the maintenance of normal respiration. The third reaction requires arginase but will proceed in the absence of the normal cell structure. (f) The failure of birds to form much urea may now be explained by the absence of arginase from the avian liver. (g) The optimum pH for the entire process is between 7 and 8. (h) Although ornithine catalyzes urea formation, it is without effect on the rate of oxygen consumption.

Ackermann has shown (2) that arginine may be converted into citrulline by bacterial guanido-desimidase (3). The reverse process, required by Krebs's scheme for urea formation, awaits demonstration in a simple, isolated system. Apparently exogenous arginine is catabolized very completely; neither citrulline nor ornithine was excreted after administering arginine subcutaneously to dogs (4).

It is now established by the work of Salaskin's laboratory that urea formed in the autolysis of liver (5) and placenta (6) arises exclusively from arginine.

Rôle of the central nervous system.—In a series of abstracts (original papers in Japanese), the conclusion is drawn that a center exists in the brain which regulates the formation and excretion of urea. It is alleged to be dual in nature, operating through the sympathetic and parasympathetic systems (7). Richet & Dublineau (8), by the piqûre method, conclude that the center is localized in a "région supérieure de la protubérance"; it is distinct, however, from the centers of polyuria, glycosuria, and thermogenesis.

In several abnormal mental states the rate of urea formation from

administered amino acids is found to proceed more rapidly; the blood urea-content also ascends to a high level (9).

Retention of urea.—Kocher & Torbert (10) have been unable to confirm the observations of Moore *et al.* (11)¹ in experiments on a healthy subject in a state of minimum-protein metabolism. Twenty-five cases of diabetic coma with urea retention have been analyzed by Lyall & Anderson (12). In agreement with Labb  they find the prognosis to be poor if the blood urea-concentration exceeds 100 mg. per cent. The cause of the retention is not clear, although renal involvement is common. The problem is fully discussed by Daniels & Touw (13) also. Urea retention in Addison's disease and other non-diabetic conditions are reported upon by Simpson (14), Will (15), and Jung (16).

Function of urea.—According to Simpson & Ogden (17) urea, specifically, is necessary for maintaining the normal beat of the elasmobranch heart. This confirms the older work of Mines.

Analytical methods.—An ingenious micro-method for the quantitative determination of urea is reported by Walker & Elsom (18). It is capable of estimating 2.5×10^{-5} mg. of urea. By its use the authors succeeded in demonstrating that the urea content of frog plasma is equal to that of the glomerular fluid.

AMMONIA

Blood.—The technique of blood-ammonia determinations has been examined in detail by Folin (19). The normal content for human blood is found to be about 0.1 mg. per cent. In consequence, Folin is now inclined to question the conspicuously low values (<0.05) reported by others.

The autolytic formation of ammonia continues to show striking differences from species to species; in some cases it is inhibited by borate and in others not; in some species hemolysis increases ammonia formation and in others it is without effect (20, 21, 22). The behavior of turtle blood is especially peculiar (22). It is clear from these studies that the adenine nucleotides are not the only precursors.

Muscle.—The formation of ammonia from the adenine nucleotides in skeletal and heart muscle is being actively investigated (23), but interpretation of the experiments is still too obscure and controversial for detailed review.

¹ Cf. *Ann. Rev. Biochem.*, 1, 300 (1932).

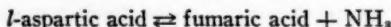
Brain.—The ammonia content of rabbit and human brain is about 0.4 mg. per cent (24). It is increased by trauma (25), is uninfluenced by lack of oxygen and narcotic gases (25, 26), and is reduced by insulin even though hypoglycemia is prevented by glucose administration (24). Riebeling (27) finds that the ammonia content of the brain in *status epilepticus* is more than twice as great as the normal value.

Kidney and urine.—The classical experiments of Nash & Benedict on the renal origin of urinary ammonia are fully substantiated by Nash & Williams (28) and Wassermeyer (29). The latter's work pertains primarily to adenine nucleotide as a precursor of the ammonia. Musella (30) has shown, in nine cases of Parkinson's disease, striking disturbances of the normal relation between pH and ammonia excretion. The ammonia quotients and Hasselbalch constants were abnormally high. The excretion of ammonia by infants, in health and disease, has been studied and reported in a long communication by Ducco (31). In all pathological cases Ducco gives due attention to the effect of pH by comparing the ammonia quotients observed with those of normal specimens of the same pH.

AMINO ACIDS

Alanine.—A promising method for the determination of alanine is described by Kendall & Friedemann (32). A micro-adaptation of the procedure, suitable for use in protein analysis, is given by Fürth *et al.* (33).

Aspartic acid.—The free energy changes for the reactions



have been calculated from the observed equilibria by Borsook & Huffman (34), and found to equal those obtained from thermal data. Aspartase, the highly specific catalyst of these reactions, has been carefully studied by Virtanen & Tarnanen (35). It is widely distributed in bacteria and the higher plants but has not been found in animal organs (35, 36).

Histidine.—Since Dakin's study of the problem in 1913, urinary formic acid has been thought to be a product of histidine catabolism. Zeyen (37) now reports that histidine administered to normal subjects did not increase the formic-acid excretion. In parenchymatous diseases of the liver, it caused a slight increase. Twenty grams of glycine (in jaundiced patients) produced a more marked effect.

Histidine is deaminized by ultra-violet light (38) to a much greater degree than other amino acids. Imidazoacetaldehyde, ammonia, and carbon dioxide are the products (39).

Proline and hydroxyproline.—Bernheim & Bernheim (40) report that these amino acids are readily oxidized by liver (Warburg technique). The catalyst is a dehydrogenase. Neither ammonia nor CO_2 is produced, and one atom of oxygen is taken up for every two mols of proline that react. A method for the quantitative determination of proline and hydroxyproline has been developed by Fürth & Minnebeck (41).

Phenylalanine.—The intermediary metabolism of phenylalanine has been carefully studied by Chandler & Lewis (42). Following four-gram doses of *dl*-phenylalanine administered to rabbits about 0.35 gm. of phenylacetic acid and correspondingly small amounts of phenylpyruvic acid were excreted. Still less of an effect followed the administration of *l*-phenylalanine. A five-gram dose of phenylpyruvic acid (sodium salt) gave 1.1 gm. of phenylacetic acid. Interpretation is difficult in view of the fact that 80 to 90 per cent of the amino acid remained unaccounted for. With phenylethylamine, on the other hand, 78 per cent was converted to phenylacetic acid. A quantitative method for the determination of phenylalanine, consisting in the colorimetric determination of *p*-nitrobenzoic acid formed by oxidation with nitric acid (Kollman), is now available (43).

*Tryptophane.*²—By the use of kynurenin as a supplement in tryptophane-free diets, Jackson & Jackson (44) have been able to show that the catabolism of tryptophane to kynurenin is irreversible. Fed as the sodium salts, *l*- and *dl*-tryptophane are found by Berg & Baugess (45) to be almost equally well absorbed from the rat intestine. The rates of absorption (mg. per hour) approximate those reported for the sodium salts of glycine and glutamic acid. An enzyme known as tryptophanase which converts the amino acid, specifically, into products as yet unidentified has been reported by Kurono, Katsume & Oki (46). The tryptophane content of blood and various organs is the subject of a study by Re & Potick (47).

From evidence gained in nutrition experiments, DuVigneaud, Sealock, & Van Etten (48) show that *d*- and *l*-tryptophane are

² [Note added to proof:] A series of papers from Kotake's laboratory on kynurenin formation were reviewed last year [*Ann. Rev. Biochem.*, 1, 304 (1932)]. Others, too late for inclusion in the present review, have now appeared [*Z. physiol. Chem.*, 214, 1 (1933)].

equally well utilized by rats. This confirms observations by Berg & Potgieter (49). Acetyl-*d*-tryptophane, on the other hand, is not metabolized even though acetyl-*l*-tryptophane gives positive results. Furthermore, it is shown (50) that *d*-cystine is not able to replace *l*-cystine [see also Lawrie (51)].

Permanent blindness, unlike xerophthalmia arising from vitamin-A deficiency, is reported by Curtis, Hauge & Kraybill (52) to develop in animals maintained on tryptophane-deficient, animal-protein concentrates.

Tyrosine.—A new and rare⁸ error in tyrosine metabolism, to which the name "tyrosinosis" has been given, is fully reported by Medes (53). The condition is characterized by an inability to carry the oxidation of tyrosine beyond the first stage. Under endogenous conditions, Medes's subject excretes about 1.6 gm. of *p*-hydroxyphenylpyruvic acid daily. Elevation of tyrosine metabolism is followed by the excretion of, first, tyrosine, later, *l*-*p*-hydroxy-phenyl-lactic acid, and finally, as the metabolism is raised still further, *l*-3,4-dihydroxy-phenylalanine. The last two compounds are thought to arise through side reactions. On a high-protein diet, the output of *p*-hydroxy-phenylpyruvic acid was doubled. It is interesting to observe that the administration of phenylalanine also increases the subject's output of *p*-hydroxy-phenylpyruvic acid. Glycine, tryptophane, and creatine are without effect. Evidence is also presented that the subject suffers a retardation in the first stage of tyrosine metabolism; a given dose of the amino acid leads to tyrosinuria, not observable in a normal individual. Finally it is shown that the oxidation of tyrosine to 3,4-dihydroxy-phenylalanine is reversible.

The results suggest that the error essentially consists in an inability to oxidize a *p*-hydroxy metabolite in the 2,5-position. A 2,5-dihydroxy derivative, once formed, is apparently fully oxidized, as indicated by the subject's ability to utilize homogentisic acid.

Ballowitz (55) has reported a case of alcaptonuria in infancy in which 1.76 to 2.51 gm. of homogentisic acid are excreted daily.

Hematopoietic function.—Using normal animals, Fontès & Thivolle (56) were able to show that tryptophane and histidine are hematogenic; significant increases in hemoglobin content and erythrocyte count were reported. Lysine, leucine, and phenylalanine

⁸ Medes has discovered only one case. Blatherwick (54) reports that 14,753 urines examined under his direction revealed a single case of alcaptonuria and none of tyrosinosis.

gave only negative results. Rats on a diet deficient in histidine and tryptophane showed a marked decrease in hemoglobin index.

In certain human anemias, tryptophane and histidine are reported to have some stimulating action on the hematopoietic tissue (57). Likewise in experimental phenylhydrazine anemia, Okagawa & Tatsui (58) have confirmed Hirazawa (59) in showing that tryptophane accelerates hemoglobin regeneration. Splenectomy abolishes the effect. It is interesting that methyl tryptophane is quite as effective as tryptophane (60).

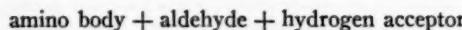
As for pernicious anemia, there is no satisfying evidence that tryptophane, histidine, or other amino acids promote hemoglobin formation (61). The work of Keil & Nelson (62) strongly suggests that the same generalization may be applied to nutritional anemia in rats.

Glycemic action.—The action of amino acids in altering the blood-sugar content and in augmenting or decreasing the response of the organism to injections of adrenaline and insulin continues to receive active investigation (63). Nevertheless, the results from different laboratories are so discordant that a detailed review of the literature would be unprofitable. One of the most striking observations is reported by Schenck (64), who contends that glycine administered to rabbits in moderate doses (2 or 3 gm.) by mouth is markedly hypoglycemic. The whole problem of amino-acid glycemia involves fundamental questions pertaining to the pharmacodynamic action of amino acids, glucogenesis, and intermediary metabolism. It deserves careful re-investigation.

On tissue respiration and deamination.—Using the organ-slice method of Warburg, Kisch (65) has studied the effect of amino acids and dipeptides on the rate of oxygen consumption of various tissues. Kidney tissue showed a more marked response than liver, the respiration of which was affected to but a small extent. The most active amino acids were alanine, phenylalanine, serine, valine, sarcosine, and phenylaminoacetic acid. The respiration of Jensen's rat sarcoma, on the other hand, was not elevated by these substances. The optimum pH range was 7.4 to 8.0. Fresh tissue slices were generally less satisfactory than older preparations.

In related investigations (66) the oxidative deamination of amino acids by various *o*-quinones was studied. The amount of ammonia formed during periods of 20 to 40 hours was measured. In the range pH 10 to pH 11 the following amino acids were partially deaminized:

glycine > serine > phenylaminoacetic acid > leucine. Alanine was not deaminized. Various *o*-quinones were used, the most effective being pyrocatechol, hydroxyhydroquinone, and adrenaline. At pH 7 to 8, glycyl-*l*-tyrosine was partly deaminized though glycine and *l*-tryptophane were not acted upon. *M*-dinitrobenzene would satisfactorily replace O₂ as the hydrogen acceptor (67). These studies are intimately related to those published in 1925 by Happold & Raper and Robinson & McCance. Studies on the oxidation of amino acids by dialuric acid (Strecker's reaction) are reported by Hill (68). The system



has been fully investigated by Lieben & Getreuer (69). Substantial evidence is advanced in favor of the formation of Schiff's base (aldehyde-amino body), with subsequent dehydrogenation by the hydrogen acceptor.

Bergmann (70) reports the synthesis of several dehydropeptides [e.g., C₆H₅ · CH = C(NH · CO · CH₂NH₂) · COOH] which were acted upon by a kidney enzyme to liberate a keto acid, ammonia, and an amino acid. The author suggests that deamination of amino acids *in vivo* may proceed through the formation and dehydrogenation of simple peptides.

Aminoacidemia.—Zirm & Benedict (71) report the results of a critical examination of present methods for amino-nitrogen determinations in blood. A new volumetric method which compares favorably with the Folin and Van Slyke methods is developed.

Four valuable papers by Becher & Herrmann (72) have appeared, in which the determination of both free and "bound" amino N is critically considered. The latter is expressed as the difference between amino-nitrogen values on trichloroacetic-acid filtrates before and after hydrolysis [see also Hülse & Franke (73)]. The authors show that most of the "bound" amino N is in the erythrocytes and is probably not of peptone or polypeptide nature. In general, the "bound" amino-N content of pathological blood varies directly as the free amino N. Normally the "bound" amino N is 1.3 to 3.3 mg. per 100 cc. of blood. It is significantly higher in acute yellow atrophy of the liver, polycythemia, and myeloid leukemia, in which the free amino N is again shown to be elevated [see also Simon & Zemplén (74)]. Shortly before death both values are very high. In certain renal lesions the "bound" amino N is sometimes disproportionately

high (72, 73). In pulmonary tuberculosis (75) and in various endocrine and joint diseases (76) the free amino N is unchanged. In severe cardiac diseases, especially with dropsy and engorged liver, Kisch (77) reports marked increases in the free amino N.

Schlossmann (78) shows that amino acids injected into the maternal circulation pass freely through the placenta and soon attain a relatively higher level in the fetal blood (8.7 to 12.1 mg. amino N per 100 cc.) as compared with 5.7 to 8.2 mg. in the maternal blood.

THE RÔLE OF THE ENDOCRINES IN PROTEIN METABOLISM

Hypophysis.—Braier (79) reports observations on five hypophysectomized dogs. During a 12-day fast the rate of nitrogen excretion was substantially less than that of normal animals similarly fasted. For the entire period the average values were 0.25 and 0.36 gm. N per kilo per day, respectively. The administration of a fixed amount of meat on the second fasting day increased the nitrogen loss during the following 7 or 8 hours from 19.1 to 46.0 mg. per kilo per hour in the five hypophysectomized animals, and from 28.1 to 78.4 in five normals. The excretion of extra nitrogen by the hypophysectomized animals is merely delayed, being extended into the ensuing 18 hours. On low-protein diets, the rate of nitrogen excretion fell to 0.14 and 0.24 gm. per kilo per day respectively. The metabolism of intravenously injected glycine proceeds more slowly in the hypophysectomized animal [Re (80)]. It is difficult to reconcile these reports of reduced nitrogen metabolism after hypophysectomy with other observations to the effect that anterior-pituitary extracts administered to normal animals decrease the rate of nitrogen excretion (81) and reduce the non-protein-nitrogen content of the blood (81, 82).

As for the calorigenic action of proteins in human hypophyseal deficiency or in the hypophysectomized animal, much uncertainty still prevails. However, the abundant clinical material studied by Fulton & Cushing (83) and the recent observations of Johnston (84) agree with the animal experiments of Artundo (85) and Gaebler (86) in indicating that the calorigenic response to protein is of normal magnitude. It is nevertheless unfortunate that in almost all experiments of this type the authors arbitrarily select a certain brief time-interval for measurement of the effect. In other instances only the time and magnitude of peak values in heat output or oxygen consumption are recorded. The total increase in heat output from the

time of administering protein until the return to basal is much more important.

A marked reduction in the basal metabolic rate in hypophyseal insufficiency seems to be definitely established (83, 85). An excellent review of the whole subject is given by Houssay (87).

Thyroid.—From observations on 11 cases of exophthalmic goiter with high basal metabolic rates (+34 to +89), Klein (88) shows that the absolute increase in calories following a protein meal is quite great, though usually less than in normals. Thyroidectomy prevents the increase in protein metabolism which is observed in normal animals after administering phlorhizin (89).

THE PLASMA PROTEINS

Analytical methods.—Apart from a critical comparison of the Wu and Kjeldahl methods, from which it is concluded that the former gives divergent and misleading values in low-protein sera (90), there are no contributions to the better-known procedures for plasma-protein determinations that require mention. Numerous clinical methods have been described. Schmitz & Wulkow (91) have used the Zeiss stufenphotometer in quantitative measurements of the amount of protein salted out from 0.1 cc. portions of serum by increasing concentrations of ammonium sulphate. By plotting light-absorption against ammonium-sulphate concentration, curves are obtained which show definite and characteristic departures from the normal in nephrosis, tuberculosis, and carcinoma. Ninety-six per cent of the 72 carcinoma and tuberculosis cases conformed to the types described. The "scopometric" method of Exton & Rose (92) employs the same principle and follows a somewhat similar procedure. Both methods permit a rapid differential analysis of the various plasma-protein fractions.

An ingenious and simple test for low-plasma protein is the specific-gravity method of Page & Van Slyke (93). An improved direct-nesslerization method is fully described by Hubbard (94). The determination of buffer value (95), oxidation by permanganate or dichromate (96), and decomposition by hypobromite (97), are used in other clinical procedures newly described.

Formation and origin.—A study of the serum-protein content and serum osmotic pressure in young animals is reported by Clark & Holling (98). The serum-protein content in puppies is shown to increase rapidly for the first 40 or 50 days, and then more slowly

until the 70th or 80th day, by which time the adult level is reached. The value at birth is about 2.2 gm. per 100 cc. of serum. The osmotic pressure of the serum increases in such a way that the linear relationship expressed by the equation

$$\frac{1}{\text{protein concentration}} = K + \frac{1}{\text{serum osmotic pressure}}$$

is observed.* As might be expected the blood pressure of the young animal steadily increases, keeping pace with the increases in protein content and in serum osmotic pressure. A related investigation is that of Swanson & Smith (99) on the total plasma-nitrogen content of rats at different ages. A similar increase in plasma-protein content is indicated.

The fibrinogen content of plasma is shown to decrease after hepatectomy (100). Except for the possibility that accelerated fibrinolysis accounts for this change, the results support the established theory that fibrinogen is of hepatic origin.

In several papers Fischer (101) has developed the thesis that serum globulin is, in reality, heparinized albumin. The theory is based upon the formation of a globulin-like precipitate at pH 5 when heparin is added to serum. Henriques & Klausen (102) now show that the theory is untenable, since the addition of heparin does not increase the true globulin content as determined by salting-out.

Phosphorus poisoning is shown to reduce markedly the serum-albumin content of dogs, without any effect on serum globulin. Ligation of the bile duct decreased the serum albumin and increased the serum globulin. From experiments on hemorrhage in rabbits, with and without return of the erythrocytes, the authors conclude that albumin is regenerated more quickly than globulin.

Proteinuria.—The incidence of albuminuria in young men (students) is reported upon by Diehl & McKinlay (103). Of 20,000 urines tested during the decade 1921 to 1930 by the nitric-acid-ring test, 5.32 per cent were positive on the first examination. The more sensitive sulphosalicylic acid test would have given, unquestionably, a much higher apparent incidence. Persistent albuminuria was observed in 0.62 per cent, and definite kidney disease in 0.34 per cent.

The well-known effect of exercise in increasing albuminuria has

*A progressive increase in osmotic pressure per gram of protein was also noted.

been studied by Hellebrandt (104), who shows that the incidence of albuminuria increased from 15 per cent in a group of 47 female subjects to 58 per cent following exercise. Of thirteen football players who played for 45 to 60 minutes, all were found by Edwards, Richards & Dill (105) to exhibit albuminuria (urine-protein content averaged 2.8 per cent).

The ability of the normal kidney to excrete foreign protein is shown by Kerridge & Bayliss (106) to be primarily a function of molecular size. Proteins of low molecular weight are readily excreted, those of larger size (M.W. $\geq 68,000$) are retained. A brief, interesting report on albuminuria of pregnancy is given by O'Sullivan (107). Eastmen (108) points out that about 25 per cent of the urine protein in eclampsia is globulin, instead of 7 to 13 per cent as in nephritis and nephrosis. Presumably the glomerular lesions are unusually severe. A remarkable renal degeneration of dietary origin is described by Cramer (109) who observed that rats maintained upon diets low in magnesium develop albuminuria within a few weeks and exhibit severe degeneration of tubules and glomeruli.

The theory that urinary albumin is toxic and may be the cause rather than the result of renal degeneration is revived by Garnier & Marek (110) to interpret many observations on uranium nephritis in dogs and rabbits. Incidentally the authors report that rabbits can be habituated to uranium nitrate. Eventually the large doses (60 mg. per kilo) have little effect, although for a period there is persistent albuminuria without nitrogen retention.

Two recent studies of extra-renal albuminuria can be mentioned by reference only (111). Both are concerned with the centrally conditioned albuminuria observed in sub-arachnoidal bleeding.

A simple, ingenious method of clinical interest for the quantitative determination of urine protein is described by Kerridge (112). Osgood & Haskins (113) have reported the results of a critical study of the heat-coagulation and sulphosalicylic-acid tests for the routine examination of urine.

Bence-Jones protein.—An exhaustive review of Bence-Jones proteinuria, with a study of several new cases, has been published by Magnus-Levy (114). The author inclines to the opinion that the weight of evidence, including his own recent observations, indicates that Bence-Jones protein is partly exogenous in origin, or that its formation by the bone marrow is accelerated by increased protein intake. Nevertheless, the subject recently studied by Mainzer (115)

excreted protein at a constant rate independent of exogenous protein metabolism. The remarkable ability of the organism to regenerate protein is demonstrated in several cases cited by Magnus-Levy in which 40 to 70 gm. of protein were excreted daily. The bone marrow is regarded as the site of origin of Bence-Jones protein and, indeed, of the plasma proteins also.

About 20 to 25 per cent of the cases of multiple myeloma show no proteinuria. It is conceivable, however, that some of these may show Bence-Jones proteinemia. Thus, Shirer, Duncan & Haden (116) add to the few cases of Bence-Jones proteinemia on record, one in which the blood contained 7.4 to 8.8 gm. of Bence-Jones protein per 100 cc. of blood. None was excreted. In the second case a value of 4.2 gm. per 100 cc. of blood was found. Finally Reimann's subject (117) had a high plasma-protein content (10.12 per cent) but did not excrete any Bence-Jones protein. Neither of the subjects of Shirer *et al.* gave evidence of renal disease. In a case studied by Ehrich (118) the kidneys showed hydronephrotic atrophy following occlusion of the tubules.

Sedimentation of erythrocytes.—Inasmuch as the sedimentation rate of the erythrocytes in citrated blood is now recognized to be largely a function of the fibrinogen and globulin content of the blood, no review of the plasma proteins would be complete without reference to this important phenomenon. Interest in the subject has so increased since the monumental work of Fähraeus (119) that hundreds of papers have been devoted to it in the past decade. Leffkowitz (120) cites 670 articles during the period 1927 to 1931.

The relationship between sedimentation velocity, red-cell volume (hematocrite reading), fibrinogen- and globulin-content are now quantitatively formulated by Snapper and colleagues (121) as follows:

$$V = \frac{45}{\text{cell vol.}} [12 (\text{fibrinogen } \% - 3.5) + 2.5 (\text{globulin } \% - 22)]$$

where V , the sedimentation rate in normal blood, is regarded as 1, and the normal percentage values for cell volume, fibrinogen, and globulin are 45, 0.35, and 2.2 respectively. The factors 12 and 2.5 are empirical. The theory of sedimentation developed by Fähraeus from Stokes's law is again treated by Bendien, Neuberg & Snapper (122). For cases of severe anemia, a correction factor I (hemoglobin index) is proposed for insertion in the sedimentation formula.

The sedimentation rate is either independent of or varies inversely as the albumin content (123). It also varies directly with the age of the subject (124), owing to a gradual increase in fibrinogen content with advance of age. Where slight inflammatory conditions are present an increase in globulin also is not uncommon. The greater sedimentation rate in women as compared with men is probably due to a higher globulin content (125). The well-known increase observed in pregnancy is apparently due to increases in both fibrinogen and globulin (126).

The value of the test in routine clinical examination, and as a diagnostic aid in tuberculosis, febrile infections, arthritis, and inflammatory conditions generally, is supported by the results of numerous investigations (127).

Nutritional hypoproteinemia.—From observations on 57 patients with evidence of malnutrition (tuberculosis, diabetes, and gastrointestinal disease) and by comparison with well-nourished subjects in hospital, Bruckman, D'Esopo & Peters (125) showed that the serum-albumin level was characteristically low. On high-calorie diets with abundant protein the serum-albumin content gradually increased. Similar findings have been reported by Weech & Ling (128), who studied 18 malnourished subjects. All were edematous on admission; there was no renal or cardiac involvement. The serum-albumin content, which averaged about 2 per cent, gradually increased after protein enrichment of the diet. Simultaneously the edema disappeared. Peters reported edema in malnutrition when the serum-albumin content fell below 3 per cent. The administration of a low-protein diet to a dog for 98 days was found by Shelburne & Egloff (129) to result in marked reduction of serum albumin and the development of massive edema. Liu *et al.* (130) studied hypoalbuminemia of nutritional origin in two patients. In forty-three cases of pellagra, Turner (131) made plasma-protein analyses which indicated a tendency toward hypoalbuminemia, the degree of which varied as the severity of the disease. It usually persisted even on high-protein diets and bore no causal relationship to the disease. The albumin determinations were of prognostic value.

There is no recent work on hypoalbuminemia in endemic nutritional edema. It is to be hoped that Youmans will supplement his recent observations (132) on seasonal nutritional edema, endemic in Tennessee, with plasma-protein determinations.

Experimental hypoproteinemia.—Plasmapheresis was described by

Abel, Rowntree & Turner in 1914. Since then the method has been used in numerous investigations devoted to studies of the plasma proteins. It is fully established that hypoalbuminemia accompanied, ultimately, by edema is a characteristic result. Since the globulin content can only be lowered by rapid bleeding (133) the rate of regeneration of globulin is apparently more rapid than that of albumin (134, 135; see also older studies of Whipple). In fact the stimulus to globulin regeneration commonly leads to over-compensation, which causes the globulin content to rise above the normal level (135, 136). Fibrinogen is replaced with extreme rapidity (133).

By determining the maximum rate at which plasma may be removed without lowering the plasma-protein content, conclusions may be drawn regarding the rate of regeneration of the plasma proteins. Application of this method to four dogs by Barnett, Jones & Cohn (137) indicated a regeneration rate of 0.4 gm. of protein per kilo per day. By a somewhat different procedure Whipple and associates (138) determined regeneration rates of 0.15 to 0.9 gm. per kilo per day. Mention is made (137) of a patient with cirrhosis of the liver who lost 10 gm. of protein daily for 8 months. This corresponds to a protein-regeneration rate of 0.10 to 0.15 gm. per kilo per day. Such studies are of importance in the clinic, where hypoalbuminemia may frequently be observed in nephritic patients, even though the renal loss of protein may not exceed 1 gm. per day (139). This is so far below the probable rate of protein regeneration by the normal subject that an impaired ability to synthesize plasma protein is clearly indicated.

Edema generally appears when the albumin content falls below 1.5 per cent (140). Since the experimental animals show no cardiac or circulatory disturbances the edema may be explained in terms of the Starling hypothesis as due to the reduction in protein osmotic pressure.

According to Field & Drinker (141) the capillary endothelium, coincident with the temporary reduction in blood pressure and plasma-albumin content, changes its permeability toward protein by permitting a direct return of protein from the tissue spaces into the blood.

Experiments on plasmapheresis do not support the hypothesis that the renal lesions in nephrosis are secondary to the hypoalbuminemia (135, 140).

The dependence of serum calcium and chloride upon protein content, qualitatively recognized for some years, has been quantitatively expressed by Darrow *et al.* (140) [cf. also Oberst & Plass (142)].

Clinical observations.—A series of papers pertaining to the plasma proteins in disease have been published by Peters and colleagues (125, 143). It is impossible to do justice to these studies in the limited space available, but attention may be drawn to certain points of interest. Both total protein and serum albumin were found to be as low in chronic glomerular nephritis (nephrotic type) as in amyloid nephrosis. Non-cardiac edema, obstinate in type, developed when the total serum protein fell below 4 per cent. Vascular disturbances were minimal. The albumin deficiency was probably referable to massive albuminuria, although other factors were involved. Fourteen of the 21 patients gave evidence of malnutrition—more specifically of protein starvation. The albumin/globulin ratio in such studies is regarded as almost valueless. The absolute concentration of serum albumin is much more important. In 38 patients with acute nephritis, low levels of albumin were again observed. Edema was always present when the total protein content fell below 4 per cent, but it often occurred when the value was almost normal. In such cases the edema was probably due to vascular disturbances associated with hypertension and increased capillary permeability. In heart failure, the serum-albumin content is also frequently reduced. The edema is often associated with some degree of albumin deficiency, which in turn is directly referable to malnutrition. The main cause of the edema is increased capillary blood pressure due to venous congestion. In the terminal states of renal disease reduced serum albumin, normal globulin, and high non-protein N are common. The edema is more closely related to heart failure than to lowered serum albumin. Malnutrition is characteristic. In general, the authors emphasize the significance of malnutrition (protein starvation) in relation to the albumin deficiency which marks these diseases. Chronic nephritis is definitely regarded as a wasting disease.

Epstein was the first to recognize the need in such cases of restoring the serum albumin to the normal level and to this end strongly advocated recognition of nephritis as a wasting disease and the abandonment of low-protein diets. The necessity of maintaining the protein intake at a fairly high level is now receiving wider emphasis (144).

All recent work confirms the fundamental observations of Epstein on hypoalbuminemia as the causal agent of nephrotic edema. Leiter's paper (145) reviews in detail the recent literature on the subject. That of Kumpf (146) on the blood proteins in renal diseases generally should also be mentioned. The relatively high proportion of "lipoprotein" in the blood in lipoid nephrosis is demonstrated in a brief communication by Macheboeuf & Wahl (147). A marked reduction of the serum-albumin in hookworm disease (148), and an increase of serum-globulin in leprosy (149) have been reported recently. Beard, Blalock, *et al.* have published several papers on the plasma proteins in shock (150).

A recent paper by Fuchs (151) describes the technique of his serum test for cancer and gives further evidence of its value in the diagnosis of malignant growths. The test is based upon the Abderhalden reaction and depends upon the remarkable specificity of the serum enzymes in digesting foreign fibrin. M. von Falkenhhausen (152) and Fuchs (151) draw attention to the need of great accuracy in performing the non-protein-nitrogen determinations required in this test. The former regards it as the best method available for the diagnosis of cancer. A curious increase in the amide nitrogen of the plasma proteins in cancer is reported by Merzbach (153).

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THE METABOLISM OF CREATINE AND CREATININE*

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Rarely have two biochemical compounds been the objects of so many investigations or provided the occasion for as much debate as have creatine and creatinine. Since the discovery of creatine by Chevreul in 1835, it and its dehydration product, creatinine, have attracted the interest of scores of chemists, biochemists, and physiologists. Publications numbering many hundreds have been reviewed in the admirable monograph of Hunter (1). Since the appearance of this valuable work in 1928, a summary of the more recent literature has been provided by Myers; while the unique rôle of creatine in muscular activity has been discussed in the monograph of Meyerhof, and in the reviews of Milroy, Lindhard, Hill, Parnas, and others.

In the following pages we shall confine our attention largely to the literature bearing upon the metabolism of creatine and creatinine, with special emphasis upon the more important publications since the appearance of Hunter's monograph. In thus limiting the scope of our review, we are deliberately excluding articles dealing with the chemistry of these two compounds, the derivatives which they yield, the chemical transformations involved in their color reactions or precipitation tests, and the many methods which have been devised for their qualitative detection and quantitative estimation. To cover adequately the chemical as well as the metabolic aspects of creatine and creatinine would require much more space than could appropriately be allotted to the subjects in a volume of this sort. It should be pointed out, however, that the structural configurations of the two compounds in question are more or less closely related to several other substances of biochemical interest. The guanidine group of the creatine molecule immediately brings to mind the structure of arginine. The presence in creatinine of the glycocyamidine ring suggests the structures of histidine, the purines, and allantoin. In so far as these chemical similarities may have any suggested bearing upon creatine-creatinine metabolism, they will be referred to later.

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THE DISTRIBUTION OF CREATINE AND CREATININE

1. *Normal distribution.*—Abundant evidence is available indicating that creatine is a constant component of the voluntary muscles of mammals, birds, reptiles, amphibians, and fishes; and of certain organisms still lower in the scale of animal life. Hunter (2) analyzed the skeletal muscles of fifteen different species of fishes and found creatine invariably present. Indeed, the author reports that in general the values obtained were higher than those for mammalian muscles. The red muscles of fishes, as of mammals, were found to carry less creatine than the pale; and fetal muscles less than those of the adult. No characteristic difference was observed in the proportion of creatine in the muscles of *Elasmobranchii* and *Teleostomi*.

Different species of animals usually show different, and more or less specific, ranges of creatine values in their skeletal muscles; but individuals of a given species have fairly uniform proportions. In general, the concentration in cardiac muscle is considerably less than in the voluntary muscles of the same species, while the amount present in smooth muscle is even smaller. In so far as the writer is aware, there are available no isolation experiments which demonstrate convincingly the presence of creatine in smooth muscle, but the analytical values obtained by the application of colorimetric methods render its existence in such tissues very probable.

In addition to the muscle tissues, creatine has been isolated from the brain and testes of vertebrates. In two analyses of Selachian nervous tissue Hunter (2) found the creatine content equal to that of the mammalian brain. Chanutin & Silvette (2) report that the skin of the rat contains a surprisingly large amount of creatine (0.11 per cent). Its presence in traces in other parts of the organism has been rendered likely by the usual tests.

The distribution of creatinine has not been so definitely determined. The small amount of the chromogenic material present in tissues, as indicated by the application of the Jaffe test to extracts, has prevented the isolation and identification of the reactive substance. Furthermore, if one assumes that creatinine alone is responsible for the color, the possibility of creatine dehydration introduces an element of uncertainty as to the reality of the existence of creatinine in a preformed state in the tissues from which the extracts are made. In experiments designed to prevent, as far as possible, the formation of creatinine from creatine, the results indicate that the former is present in muscle, and probably other tissues, in amounts

varying from 2 to 10 or 12 mg. per 100 gm. of tissue. Even these values, at least in some cases, may be too high. In the urine of mammals creatinine is quantitatively an important component. In the excreta of birds, reptiles, and certain fishes (White), creatinine is said to be largely replaced by creatine. According to Schumann (2), considerable creatinine (3.5 to 5 mg. per cent) is present in human sweat.

The occurrence of creatine and creatinine in the blood has been the subject of debate for a number of years [Hunter (1)]. The evidence points to the fact that creatine is probably present to the extent of about 4 mg. per 100 cc. of normal laked human blood. Comparable amounts in the whole blood of other mammals are usually indicated by colorimetric methods. Similar procedures show an apparent creatinine content ranging from about 0.8 to 1.8 mg. per 100 cc. of whole laked mammalian blood. With unlaked blood Folin & Svedberg obtained very uniform normal values amounting to slightly more than 1 mg. per cent. The authors demonstrated that the apparent creatinine is equally distributed between the corpuscles and plasma. In 1927, Gaebler & Kelch reported the isolation of creatinine as its potassium picrate from normal and nephritic blood specimens. For ox blood the isolated creatinine amounted to 0.37 to 0.48 mg. per 100 cc. of blood. In a case of human nephritis 10.6 mg. per 100 cc. of blood were separated. High values were observed also in the blood of dogs after double nephrectomy. The authors call attention to the fact that both normal and retention blood samples contain chromogenic substances other than creatinine. More recently, Gaebler has stated that creatinine does not exist as such in blood, but is derived from a substance, other than creatine, which is capable of yielding creatinine in isolation experiments. Whether the creatinine-yielding material is the precursor of urinary creatinine is not known. It appears to be distributed about equally between the corpuscles and the plasma. Gaebler's findings are in accord with the earlier observations of Behre & Benedict, who expressed the view that preformed creatinine is not present in blood in detectable quantities.

In contrast to their wide distribution in vertebrates, creatine and creatinine apparently are not components of invertebrate tissues.

2. Factors which influence the distribution of creatine.—Various factors such as age, the administration of exogenous creatine, fasting, and disease may modify the normal distribution of creatine. Ontogenetically, creatine makes its appearance very early in embryonic life, and progressively increases in concentration until the

adult level is attained sometime after birth. Chanutin (3) finds that in the rat this occurs between the 30th and the 40th day. At this age the animals have reached chemical maturity in so far as their tissue creatine is concerned.

According to Chanutin (2), the administration to rats of widely different diets is without influence upon the creatine content of the entire eviscerated animals. This is said to be true whether the creatine is calculated on the moist tissues, or on the dry, fat- and ash-free structures. The author employed diets containing 25 to 75 per cent of casein, edestin, or gelatin, or 85 per cent of dried extracted beef. Double nephrectomy likewise failed to induce detectable alterations in the creatine content of the carcasses. Earlier experiments of Chanutin (1) had shown that the prolonged feeding of a diet containing 2.6 per cent of creatine fails to influence appreciably the percentage in the muscle, heart, brain, and testes; but leads to increases in the liver and kidneys. In mice, moderate storage of creatine in the muscle tissue appears to occur somewhat more readily, but saturation is reached rather promptly (Chanutin & Beard). When rats are fed larger proportions of creatine (5 to 10 per cent) for a single day, marked increases are observed in the muscles and heart, as well as in the liver and kidneys [Chanutin & Silvette (1)]. A striking observation in this connection is that continued creatine feeding is accompanied by definite decreases in all of the tissues. Furthermore, when storage has been induced, the withdrawal of the substance from the diet is followed by a rapid disappearance, during the first day, of the excess in the liver and kidneys, and only a moderate loss from the muscle tissue. The administration of creatine to rats which previously have been fasted may lead to increases of 30 per cent in the muscles as compared with the control level. Evidently, the ability of rats to store creatine is dependent upon several conditions, the full significance of which is not yet clear.

Interesting observations have been made by Chanutin & Silvette (1) regarding the behavior of muscle creatine in starving rats. These authors noted a progressive rise in the percentage of creatine which was roughly commensurate with the loss in weight. The increases varied from an average of 3.9 per cent in the rats whose body weight losses did not exceed 10 per cent, to an average of 25.6 per cent for the group which experienced declines of 30 to 40 per cent. The kidneys also yielded high values for creatine probably as a result

of the concomitant creatinuria. A relatively sharp rise in muscle creatine is said to have occurred just preceding death. Palladin & Epelbaum (3), using cats, report that starvation increases the muscle creatine. Later the creatine diminishes, but still remains higher than in normal animals.

Similar increases in creatine were observed by Chanutin & Shearer in the moist structures of whole eviscerated rats. However, when the creatine was calculated on a moisture-, fat-, and ash-free basis remarkably uniform values were found throughout all stages of fasting.

The effect of disease upon the distribution of creatine is met with most often, as might be expected, in conditions involving the muscular system. Bodansky, Schwab & Brindley have described the results of creatine determinations made upon nine different muscles removed at autopsy from a case of generalized myositis fibrosa. The values obtained were in all cases extraordinarily low, as contrasted with the creatine content of normal human muscles (Bodansky), and ranged from 160 to 324 mg. per cent. In several samples the creatine values were less than half the normal. Goettsch & Brown have recently reported marked decreases in the creatine content of skeletal muscles obtained from rabbits with nutritional muscular dystrophy. In the last stages of degeneration both the white and red muscles show 110 to 250 mg. per cent. On the other hand, neither the heart nor the brain suffers demonstrable loss of creatine.

Abelin & Spichtin state that the feeding of thyroid substance to rats produces a pronounced diminution in the creatine content of voluntary muscles and liver. The condition is accompanied by a profound depletion of the glycogen stores.

The brain of the guinea pig is said to contain considerable creatine, and the amount present is not altered by severe scurvy (Palladin & Ssawron).

THE CHEMICAL STATE OF CREATINE. PHOSPHOCREATINE

Doubtless the most fundamental contribution made to creatine-creatinine metabolism during the past decade is the discovery by Fiske & Subbarow of the chemical nature of the creatine complex present in muscles. In April, 1927, these authors (1) announced that voluntary muscle contains an unstable compound of creatine and phosphoric acid, which is hydrolyzed by stimulation, and resynthesized when the muscle is permitted to recover. At about the same time, Eggleton & Eggleton (1, 5) demonstrated the presence

of a very labile form of organic phosphate, which they named "phosphagen." They suggested (1) that it might be either a phosphoric ester of glycogen, or a precursor of both lactic acid and "lactacidogen." In a slightly later paper, the same authors (2) expressed the opinion that the compound was of the nature of a hexose-phosphate. They emphasized, however, that "phosphagen" plays an important rôle in the chemical mechanism of contractility. It remained for Fiske & Subbarow to propose the structure and point out some of the physiological properties of phosphocreatine. This they did in a preliminary communication (2) in February, 1928. The complete report appeared the following year (3). In the meantime, Eggleton & Eggleton (3) had also demonstrated the presence of phosphocreatine in muscles, but were not certain whether the substance was identical with "phosphagen" or a decomposition product of the latter. Subsequently, they (4) suggested that "phosphagen" probably consists of phosphocreatine in combination with some colloidal material.

According to Fiske & Subbarow (2, 3), phosphocreatine is made up of one molecule of creatine and one of phosphoric acid. It yields a crystalline secondary calcium salt which is represented by the formula $C_4H_8O_5PCa \cdot 4H_2O$. In the muscle cell it probably exists wholly as the secondary potassium salt. In alkaline solutions phosphocreatine is rather stable, but on passing to the acid side of neutrality there occurs a gradual increase in the hydrolysis velocity which follows, more or less closely, the rise in hydrogen ion concentration.

It does not follow from the foregoing observations that free creatine does not exist in resting muscle tissue under aerobic conditions. Eggleton reports that about 20 per cent is in the free state during rest, and that stimulation to fatigue trebles the amount. According to Masayama & Riesser, the injection into rabbits of tetrahydro- β -naphthylamine or caffeine causes a marked increase in the free creatine of skeletal muscles, accompanied by a corresponding decrease in phosphorus-bound creatine. These investigators find that the ratio phosphagen is constant for different muscles of a given rabbit, though glycogen the absolute amounts may vary. Isolated muscles, placed in oxygenated Ringer's solution, are said to lose both phosphagen and glycogen, but the quotient remains the same. In the absence of oxygen, phosphagen disappears more rapidly than does glycogen, and the quotient declines.

Several papers on the distribution of phosphocreatine have been reviewed by Palladin. Ferdinand & Feinschmidt, and Palladin & Epelbaum (1), report that the phosphocreatine content of the rapidly contracting pale muscles is greater than that of the slower-moving red muscles. According to Palladin, Kudrjawzewa & Ssawron certain types of intoxication, notably phosphorus poisoning, increase considerably the phosphocreatine and creatine content of both pale and red muscles. The effects of avitaminosis are said to be variable. In the final stages of scurvy, the phosphocreatine of the skeletal muscles diminishes. In polyneuritis, an increase or decrease may occur depending upon whether the deficiency is acute or chronic [Palladin & Epelbaum (2)].

Additional evidence of the important rôle of phosphates in creatine metabolism is presented by Brown, Imbrie & Jenkinson (1-3). These authors find that when the creatine content of muscle is artificially increased by the introduction of creatine into the duodenum, a rise in the acid-soluble phosphates also generally occurs. Furthermore, creatine administration induces a temporary fall in the output of urinary phosphates which is proportional to the extent of creatine retention, and is rendered more pronounced by the administration of parathyroid extract.

The discovery of phosphocreatine in the skeletal muscles has led to a search for it in other types of muscle tissue, and in a variety of different species. Vollmer finds it to be present in cardiac muscle. The concentration in the ventricles is said to be much greater than in the auricles. According to this investigation, 75 per cent of the total creatine of the heart is in combination with phosphoric acid. Phosphocreatine has been reported also in the smooth muscles, testes, and spleen of mammals and birds (Ferdinand & Feinschmidt, and Zanghi). In addition, its presence has been observed in reptiles, amphibians, and fishes. Clark, Eggleton & Eggleton found it in the heart of the tortoise. Duliére and Ochoa, Grande & Peraita, and many others have demonstrated its presence in frog muscles. Zagami states that the muscles of strong swimming fishes carry 0.20 to 0.58 mg. per cent of phosphocreatine, while motionless or slowly moving fishes have 0.09 to 0.22 mg. per cent. In the dogfish (*Squalus sucklui*), about 10 per cent of the total muscle creatine is present as phosphocreatine according to White. Kisch reports that the electric organ of the torpedo contains approximately as much of the compound as do the skeletal muscles of this species.

In addition to the organs and tissues indicated above, phosphocreatine has been detected in nervous tissue by Gerard & Tupikow, and recently has been reported as a component of human tumors by Boyland. One-fourth to one-third of the creatine of tumors freshly excised from rats is said to be in combination with phosphoric acid. Apparently, phosphocreatine is present wherever creatine occurs. Moreover the former represents the active or effective chemical state of the latter. It is of interest to note that in invertebrates, which are believed to be devoid of creatine, phosphocreatine performs the physiological functions discharged in vertebrates by phosphocreatine [Kutscher & Ackermann, and Lundsgaard (3)].

THE ORIGIN OF CREATINE

Biochemical literature records numerous attempts to discover the origin of creatine. Such experiments usually have involved the feeding or injection of substances known to occur in the body, or of synthetic products related in chemical structure to creatine or its anhydride. Publications which appeared before 1928 are critically discussed in the monograph of Hunter (1). During the past few years renewed interest has been manifested in this problem, and a number of significant papers have come to hand. These are outlined below.

1. *Arginine*.—This amino acid early attracted interest as a possible precursor of creatine, doubtless because it is the only known protein component which contains the guanidine group. The majority of such studies have failed to provide evidence for a direct transformation of exogenous arginine into creatine or creatinine. The problem appeared in a new light, however, following the observations of Benedict & Osterberg upon dogs, and of Rose, Ellis & Helming upon man, that the oral administration of constant quantities of creatine leads to a very gradual increase in creatinine excretion, which may not attain a maximum until after the lapse of several weeks. In view of these findings it appeared possible that the synthesis of creatine from arginine might also be a very slow process. Accordingly, Hyde & Rose administered arginine to a young man and a young woman for six and eight weeks respectively. The subjects received daily doses of the amino acid equivalent to 1 gm. of creatine. Despite the large intakes, representing in the two individuals a possible excess production of creatine amounting to 42 gm. and 56 gm. respectively, no increased excretion of either creatine or creatinine occurred. Data of similar import were secured by

Grant, Christman & Lewis in experiments upon dogs. These authors state that the "oral administration of arginine for a period of 35 days failed to influence the excretion of urinary creatine or creatinine, although exogenous creatine in small amounts resulted in prompt increases in both catabolites."

The results of the foregoing investigations seem to demonstrate that the formation of urinary creatine and creatinine is not stimulated by exogenous arginine. Nor is the creatine content of the entire animal altered by the administration of rations varying widely in the proportions of combined arginine. Thus Chanutin (2) has shown that rats receiving gelatin or edestin (high in arginine) have no greater content of creatine than do animals upon diets of casein (relatively low in arginine). Even more pertinent are the results obtained by Brown & Luck. These investigators found that phosphoarginine, which unlike arginine is not attacked by arginase, fails in mice to undergo conversion into phosphocreatine, creatine, or creatinine. In the words of Hunter (1): "So large a body of almost purely negative evidence leads one rather forcibly to suspect that, if creatine is related to arginine at all, its mother substance must be not the free amino acid, but the still-combined arginine of the muscle or other protein."

It should be borne in mind that the experiments outlined above do not exclude the possibility of creatine formation from some precursor *through arginine as an intermediate*. Indeed, available evidence indicates that in mammals, at least, arginine itself may be synthesized (Scull & Rose). The important fact is that creatine production is not exaggerated by excessive arginine administration. This distinction is not infrequently overlooked by writers on creatine-creatinine metabolism.

2. *Glycine*.—A new source of creatine has been proposed in recent years by Brand and his associates (1-3). These investigators find that patients with progressive pseudohypertrophic muscular dystrophy manifest marked creatinuria, and promptly and almost quantitatively excrete orally administered creatine. Such individuals appear, therefore, to be particularly well suited for studies of the origin of creatine, since the latter, if formed, would be largely or entirely eliminated in the urine. To several patients, amino acids and other materials were administered orally in amounts varying from 1 to 2 gm. of nitrogen daily. Glycine was found to induce a 40 per cent increase in creatine excretion as compared with the control level.

During the experiments, the sulphur output dropped, and the slight rise in urinary nitrogen was less than could be accounted for by the nitrogen of the glycine. Evidently, the extra creatine did not originate in excessive tissue disintegration. As further evidence of a direct transformation of glycine into creatine, the authors point out that the administration of benzoate, which creates a demand for glycine for detoxication purposes, was followed by a fall in creatine excretion. Compounds other than glycine were much less effective. Slight increases in creatine were induced by the feeding of sarcosine, alanine, and arginine; but nucleic acid, glutamic acid, histidine, tyrosine, and cystine were practically without influence. Betaine induced a temporary increase followed by a drop below the control level. Gelatin, rich in glycine, greatly enlarged the creatine output, while edestin exerted only slight action. In this connection reference should be made to a paper of Sasaki, in which the author reports the *in vitro* synthesis of glycocyamidine by heating a mixture of glycine and urea with glycerol in a sealed tube, and suggests the possible physiological application of the reaction.

Recently, Thomas, Milhorat & Techner have confirmed and extended the investigations of Brand in experiments upon three cases of progressive muscular dystrophy, and three of pseudohypertrophic muscular atrophy. They find that, after some weeks, the creatinuria begins to decrease, despite the continuance of glycine feeding, until it reaches the former control level. Coincidently, there is a rise in the output of creatinine, and a remarkable improvement in the clinical symptoms of the patient. The sensation of fatigue diminishes, and the function of certain muscle groups improves to the extent that activities formerly impossible can now be performed.

Thus far, the apparently specific production of creatine from glycine has been noted only in the pathological conditions indicated above. Zwarenstein, and Christman & Mosier observed no influence of this amino acid upon the hourly output of preformed creatinine in normal man.

3. *Miscellaneous sources.*—A number of papers have appeared recently from Abderhalden's laboratory dealing with the relation of purines, histidine, and several other biochemical products to creatine-creatinine formation. Abderhalden & Buadze (1-3) report that the feeding of nucleic acid or histidine leads to pronounced increases in the output of total creatinine. Hydantoin and N-methylhydantoin behave similarly. On the other hand, allantoin, uric acid, methylimid-

azole, and uracile are without effect. The authors report further that the administration of globin (hemoglobin), either entire or after hydrolysis, induces an increased elimination of total creatinine. The removal of arginine and histidine from the hydrolyzed protein destroys the creatinogenic action of the material. The return of arginine to the food is without influence, but the addition of arginine and histidine restores the hydrolyzed globin to its original effectiveness. They believe that the relationship is a direct one in which the imidazole ring of the purines or histidine is transformed into creatine (or creatinine). In confirmation of this idea, they report that the same transformations occur when the compounds in question are mixed with minced tissues.

Schumann (1) takes issue with Abderhalden & Buadze on the ground that the very small increases in total creatinine observed following the administration of 10 gm. doses of nucleic acid, or 1 gm. quantities of histidine hydrochloride, are insignificant, and probably are associated with the rise in total nitrogen output. In reply, Abderhalden & Buadze (4) present additional data, and point out that in their experiments the feeding of nucleic acid and histidine did not stimulate nitrogenous metabolism. Furthermore, they say that when the latter is increased by the oral administration of 15 gm. of glycine, or by the injection of small doses of phlorhizin, the output of total creatinine remains unaltered. Finally, Abderhalden & Buadze (5) have reinvestigated the relation of histidine to creatine-creatinine metabolism, and report that both the dextro and levo forms are effective. The dose of the amino acid necessary to produce an appreciable rise is rather large (5 to 8 gm.). This, according to them, accounts for the failure of Schumann to obtain positive results.

It is evident from what has gone before that our knowledge of the origin of creatine (or creatinine) is in a very unsatisfactory state. But if one is to accept seriously the paper of Beard & Barnes the situation becomes chaotic. These investigators report that the creatine content of the muscles of young rats may be increased by the feeding of arginine monohydrochloride, histidine, glutamic acid, aspartic acid, cystine, tyrosine, *dl*-phenylalanine, *dl*-alanine, *dl*-valine, glycocyamine, choline hydrochloride, casein, and edestin. The amino acids listed above are said to augment greatly the output of creatinine in the urine of adult rats; and, in so far as they were tested, in human subjects as well. Without questioning the accuracy of the authors' observations, one is justified in assuming that the results are

not to be attributed to a direct transformation of the compounds into creatine or creatinine. To make any other assumption would be quite unreasonable, it seems to the writer, in view of the wide dissimilarity of the compounds fed. The doses were extraordinarily large, amounting in most cases to 1 gm. for a rat weighing 40 to 55 gm. In such proportions the acids may have exerted toxic effects. Furthermore, in order to induce the animals to consume the amino acids, the food cups were removed from the cages for several hours. Young rats are very sensitive to starvation, and the creatine-creatinine changes may have been due in part to this factor. But whether the foregoing suggestions suffice to account for the findings of Beard & Barnes or not, the apparent stimulation in creatine formation and creatinine elimination certainly must have been of a non-specific character.

Because of the confused state of available information concerning the origin of creatine, no attempt will be made at this time to correlate or harmonize the conflicting data. To do so would involve an apparently hopeless task. Certain observations, however, may not be out of place. The discovery of phosphocreatine and its functions appears to justify the belief, held by some for many years, that creatine is an anabolic product which serves as an indispensable component of the muscle cell, and is not a catabolic end-product derived from a certain type of protein metabolism. This is expressed by Hunter (1) as follows: "Creatine, in fact, is probably not a waste product, but an essential tissue constituent with a special function. Its rate of production is therefore in all likelihood regulated by an internal demand, and it is not to be expected that it should be accelerated by an excessive supply of precursors, any more than the production of adrenalin or thyroxine would be increased by the administration of a dose of tyrosine." A similar view was adopted by Folin & Denis in 1914. These investigators regard creatine as a part of living protoplasm, and state: "The reason why it has been found impossible to trace the formation of creatine and creatinine to any constituent of the food is that it is not possible to increase the mass of the tissues by feeding, for . . . creatine is synthesized only in connection with the growth or renewal of the protoplasm." Like conclusions have been expressed by the writer on several occasions (cf. Rose & Cook, and Hyde & Rose).

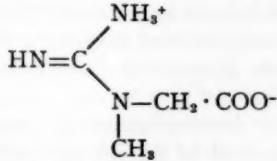
We are thoroughly aware of the fact that the point of view indicated above is rejected in no uncertain terms by many students of creatine and creatinine, who prefer to regard creatine as one of the

end-products of exogenous protein catabolism. The problem is an exceedingly complex one involving a number of unexplained factors such as variations in the powers of retention, age and sex of the subjects, the carbohydrate supply, and probably many other as yet unrecognized influences. Under the circumstances, the reviewer cannot escape the conviction that the observed increases in creatine or creatinine in the tissues or urine of normal adults following excessive feeding of particular diets may be interpreted eventually in a satisfactory fashion without necessitating an acceptance of the doctrine of an almost unlimited creatine production from widely different exogenous sources. Perhaps a more satisfactory approach to the problem of the origin of an anabolic substance would be to limit the intake of a suspected precursor below the required level, rather than to add excessive quantities to a diet already carrying sufficient amounts to meet the demands of synthesis.

THE FUNCTIONS OF CREATINE

One of the most remarkable developments of recent years is the discovery of the part played by creatine in muscular contraction. It is not our purpose to discuss this problem, inasmuch as it is covered elsewhere in this volume. Furthermore, reference has already been made to several excellent reviews of the subject. The important observation of Lundsgaard (1, 2) that poisoning with monoiodoacetate inhibits the production of lactic acid during muscular work, opened the way for an appreciation of the rôle of phosphocreatine. While all of the steps are not yet entirely clear, it seems that in the normal muscle the first change, and the one which is primarily responsible for the contraction, is the breakdown of phosphocreatine into its components.

Very little change in reaction occurs in muscle during the contraction and recovery phases. This is due, at least in part, to the buffering action of phosphocreatine, which constitutes a second important function of the compound. As pointed out by Fiske & Subbarow (3), free creatine doubtless exists in the form of an internal salt.



In phosphocreatine, present in the tissues probably as a secondary salt, there is an acid group which is about equal in strength to that of a lower fatty acid. This may indicate that the carboxyl group is free. But regardless of theoretical considerations, the hydrolysis of phosphocreatine is actually observed to liberate considerable base [Fiske & Subbarow (3); Mackler, Olmsted & Simpson; and Meyerhof & Lipmann].

In addition to the functions of phosphocreatine in muscle, Hill & Mattison, and Peabody & Hill have reported that creatine administered either subcutaneously or orally produces hypoglycemia in fasting dogs but not in fasting rabbits. Similar studies upon human subjects have been made by Koplowitz (1, 2). The latter reports that the blood-sugar values of diabetics may be reduced by the oral administration of a single dose of creatine. The effect is said to be pronounced only when the fasting level amounts to 200 mg. per cent or more. If, however, the patient is fed creatine three times daily for several days until the tissues become saturated, the oral administration of an additional amount is said to induce a pronounced increase in blood sugar. In normal individuals no significant alterations were observed following a single dose or the prolonged feeding of creatine. The blood sugar values reported by Koplowitz are so variable that one is tempted to suspect that unrecognized factors are responsible for at least some of the findings. Koplowitz (3) also reports that the injection of insulin induces in diabetics a fall in the creatine content of whole venous blood, which is followed by a rise as the sugar returns to a higher level. Very recently Mystkowski has pointed out that creatine retards, and creatinine accelerates, the enzymatic hydrolysis of soluble starch and glycogen.

THE FATE OF CREATINE. THE EXCRETION OF CREATININE

For a number of years the physiological relation of creatine to creatinine has been the subject of much controversy. The observation that the feeding of moderate doses of creatine results in its partial or complete disappearance without influencing the output of creatinine appeared to indicate that the dehydration of creatine does not take place in the body, and that, despite the similarity in structure of the two compounds, they are in fact relatively independent of each other in metabolism.

With the discovery that creatine can be transformed into creatinine by the organism, much of the difficulty inherent in the problem

has been removed. Abundant evidence for such a change is now available. The earlier data are reviewed by Hunter (1). The most striking proof of creatinine formation from ingested creatine is that provided by Benedict & Osterberg. These authors found that the daily feeding of creatine to dogs for periods of five to ten weeks leads to a very gradual increase in the output of creatinine. In one typical experiment, the rise in urinary creatinine was manifested first during the second week, and reached a maximum during the tenth week, at which time the output was 33 per cent above the level of the control period. Unchanged creatine first appeared in the urine on the tenth day of creatine feeding, and was relatively constant in quantity from the fourth week to the end of the periods of creatine administration. When the creatine feeding had been discontinued, creatine disappeared from the urine after the first day. On the contrary, the creatinine excretion diminished very gradually, and after seven weeks was still above the control level. The authors point out that of the retained creatine (i.e., creatine not eliminated as such) 29.1 to 34.2 per cent in the three experiments reappeared in the urine as extra creatinine. They also emphasize the fact that the change of creatine into creatinine is one of the slowest metabolic processes known, and probably is not a direct transformation but involves one or more intermediate reactions.

Investigations similar to those described above were conducted by Rose, Ellis & Helming upon two human subjects, one a male and the other a female. In each experiment creatine was administered for seven weeks. The results are in remarkable agreement with the data of Benedict & Osterberg. The same progressive increase in output of creatinine was observed in both cases. In the male subject, none of the creatine reappeared unchanged in the urine, but 33 per cent was recovered as extra creatinine. In the female, creatine was excreted in traces during the fore periods, but the output was greatly increased by creatine feeding. Of the retained creatine, 42.2 per cent reappeared as extra creatinine.

In view of these results there appears to be little justification for doubting that urinary creatinine can be formed from tissue creatine. On the other hand, it does not follow necessarily that creatinine is the sole end-product of creatine catabolism. It is a striking fact that both the dog and man dehydrate approximately one-third of the creatine administered in the diet. This uniform behavior led Benedict & Osterberg to state: "Our experiments seem to demonstrate almost conclusively that creatinine is but one of the end-products of creatine

metabolism." Evidence for the disappearance of creatine other than by transformation into creatinine has been provided also by Chanutin & Silvette (2). These authors calculated the creatine balance of nephrectomized rats which had received large doses of creatine, and found that much of the substance could not be accounted for. They attribute the loss to destruction by the organism. If destruction does occur, no information is available as to the intermediates or end-products of the process. In this connection it is of interest to note that according to Linneweh putrefactive bacteria transform creatine into methylhydantoin, which previously had been shown to yield sarcosine under the influence of micro-organisms. Obviously, this finding does not imply that similar reactions are accomplished by the animal body; nor is bacterial destruction in the intestine an adequate explanation for the recovery as creatinine in feeding experiments of only one-third of the retained creatine (Benedict & Osterberg, and Bollman).

The output of creatinine for a given individual on a creatine-creatinine-free diet is remarkably uniform from day to day. The prolonged feeding of a protein-free ration is accompanied by a constant elimination (Deuel and associates). The ingestion of 200 gm. of meat daily is said to be without influence on creatine-creatinine excretion, though the consumption of excessive quantities is followed by a rise in the output of creatinine but not of creatine [Eimer (1)]. Data regarding the effect of high-protein, creatine-creatinine-free diets are not uniform. Beard is of the opinion that both creatine and creatinine are products of exogenous protein metabolism, and as such are increased in output by raising the protein intake. Bollman, on the other hand, finds that the administration of high-protein diets to dogs already receiving creatine greatly reduces the amount of creatine recovered as such, and after about two weeks leads to a rise in creatinine. He interprets his findings as indicating that a high protein intake favors the dehydration of creatine.

In the past, most investigators have believed that the elimination of creatinine, in the absence of exogenous creatine and creatinine, is proportional to the mass of muscle tissue; and hence for individuals of average build is roughly proportional to body weight. Evidence in support of this conception has been presented recently by McClugage, Booth & Evans. Well-developed muscular systems were found to be associated with relatively high creatinine coefficients, while those subjects which had poorly developed muscles, or were obese, showed correspondingly lower coefficients. Data of similar import were

presented by Hodgson & Lewis. It is well known that women usually excrete relatively less creatinine than do men. In athletic women, however, the coefficients were found to be of the same order of magnitude as in men. Evidently, this generally observed difference between men and women is not a sexual one *per se*, but is dependent upon the degree of muscular development. Garot states that the output of creatinine in children is proportional to the muscular mass. On the other hand, Daniels & Hejinian believe that in infants the relation of the preformed creatinine to the length (height) of the child gives a more nearly accurate estimate of physical development than is shown by the creatinine-weight coefficient. Beard finds that the correlation coefficients between the excretion of creatinine-nitrogen and the body weight, surface area, or height of a large number of students affords little if any evidence for a relationship between the variables measured. Chanutin & Kinard made a statistical analysis of data secured in the dog, rat, rabbit, and guinea pig, and found no correlation between the creatinine elimination and the creatine content of the muscles.

Divergent views are reported in the literature concerning the influence of muscular activity upon the creatine content of the tissues and the creatinine output in the urine. Two papers bearing upon this problem have appeared during the past two years. Eimer (2) observed that the creatinine excretion during sleep is less than during the waking hours. Prolonged rest in bed is said to lead to a considerable decrease in the creatinine output of healthy human subjects. Estimations at frequent intervals show a pronounced rise shortly after muscular work with a subsequent, partially compensatory fall. The output for the total working period is greater, however, than for the same subject at rest. According to Kácl, work induces changes in the proportions of creatine and creatinine in the blood. Immediately after the completion of exercise a very slight increase in creatinine may be noted, but the rise continues and reaches a maximum one hour later. On the other hand, the creatine is said to be augmented by nearly 0.5 mg. per cent immediately after work, and decreases during the following hour. The values of both return to normal within three hours.

Under conditions which induce full renal activity, the creatinine excretion is directly proportional to the plasma concentration (MacKay & Cockrill). The excretory rate is much greater than that of urea (Cope). Comparative studies by Jolliffe & Smith (1, 2) of the

urea and creatinine clearances (i.e., volume of blood cleared of urea or creatinine per minute by the kidneys) indicate that the urea clearance averages 53 per cent of the simultaneous clearance of creatinine when the latter substance has been administered to raise the blood level. The clearances of both urea and creatinine are reduced in dogs which have been fasted or fed a low-protein diet, but are increased more than 100 per cent on an exclusive meat diet. According to Descombes, creatinine is eliminated by glomerular filtration followed by concentration through reabsorption of water. The total daily output in nephritis is regarded by Crawford as a serviceable prognostic test.

Space does not permit a discussion at this time of the various forms of creatinuria. These interesting metabolic peculiarities will be considered in a subsequent review.

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MINERAL METABOLISM—CALCIUM AND MAGNESIUM*

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CALCIUM

Calcium metabolism continues to occupy the first place in mineral metabolism. The work of compiling material has been lightened by reference to *Nutrition Abstracts and Reviews* (1). General reviews of the physiology of minerals are available in monographs by Peters & Van Slyke (7), Klinke (4), Sherman (8), Katase (3), The White House Conference (9), and Needham (5). Noorden (6) has given attention to the recent work on mineral metabolism. All of these include calcium and phosphorus. Reviews by Cantarow (54) and György (86) bring up to date many phases of calcium metabolism. The main interest centers in the state of calcium in blood and its relation to bones and teeth, the action of vitamin D and the parathyroid glands, and the calcium balances, especially in relation to phosphorus metabolism.

ANATOMY

Previous work on the mineral content of the body, blood, bones, cartilage, muscles, heart, arteries, nerves, liver, skin, and organs, and of fetus and milk, in health and disease, has been summarized by Heubner (2). The calcium and phosphorus at birth and throughout the suckling period have been determined for various animals (189, 265). Sherman & Booher (219) have demonstrated that body calcium of the rat is proportional to calcium intake. Rest or exercise, or calcium furnished parenterally, had little effect (13). The effects of the heavy metals and arsenic and iodine on the calcium content of the organism have been examined (255).

The calcium content of the muscle of normal rats has been given and, in rickets, the diminution of calcium and the altered distribution of phosphorus described (56, 97). In parathyroidectomized dogs, decreases in the calcium of the muscle have been reported (115); in

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parathyroidectomized rats, increases (51). The heart-muscle calcium of thyroidized animals has been recorded (182). The increase of calcium in the heart and kidney of chicks (239) and of rats (139) under the influence of irradiated ergosterol has been shown. The calcium and phosphorus in the brain of rats were normal in those with parathyroid tetany, and decreased in those with rickets (102).

The calcium and potassium content of skin has been reported as follows: for normal guinea pigs (41); for rabbits after injections of calcium salts and irradiation (241); after thyroparathyroidectomy (184); after large doses of X-ray (11); and in tuberculosis (99). K/Ca has been determined in lymph glands (242), and in various tissues as a measure of growth rate (123).

BLOOD SERUM

The blood cells contain traces of or no calcium (83, 204, 228, 229). Serum calcium has been extensively reviewed (7, 54, 249). Over five hundred references (1923-28) on serum calcium in humans, in health and disease, have been cited (100). The accepted values for adult serum are close to 10.5 ± 0.5 mg. per 100 cc. (45); for infants, 11.2 (21). The value in late pregnancy is 9.5 (10, 169, 170, 179, 262). The plasma calcium has been reported as higher (180) and lower (54) than that of serum (249).

Forms of calcium.—The calcium in blood serum is at present mainly discussed under the heads of (a) non-diffusible, (b) diffusible, and (c) ionic.

a) By ultrafiltration or dialysis against known calcium solutions or those free from calcium, the non-diffusible calcium is found to represent from 60 to 40 per cent of the total. It depends also upon pH, pressure, temperature, type of membrane, and lipoids (7, 54, 79, 249, 260). Additions of CaCl_2 to serum increase the non-diffusible portion also, so that the normal proportion of each is present (50, 227). The non-diffusible fraction can roughly be identified with that held by protein. Scholtz (207) has reported a colloidal complex of calcium phosphate.

b) The inorganic compounds of phosphate and bicarbonate largely constitute the diffusible group, with the addition of citrate-like compounds (Greenwald, 1926) probably produced by parathyroid. New experiments have been reported (4, 98); contra (77).

c) No reliable method is available for the determination of calcium-ion concentration. The question whether serum is saturated

with regard to calcium turns on this point (7, 249). Of the total calcium, from 40 to 50 per cent is unexplained.

Serum calcium, phosphorus, and protein.—Serum calcium varies with serum protein. Peters & Eiserson showed that this could be evaluated by a simple equation, and that the residual calcium varied inversely as the phosphate, and that both could be combined in a single equation, for adults (7). The validity of this thesis has been questioned (80, 179, 234). Carbohydrate ingestion lowers phosphorus but does not usually raise the calcium, and both calcium and phosphorus may rise or fall together, irrespective of the protein change (64, 190, 259).

Serum calcium and potassium.—Much interest has been shown in the ratio of potassium to calcium because of their well-known reciprocal relation to irritability. Studies have been made in relation to various species (267), potassium ingestion and injection (271, 272), injection of calcium (270), blood lactic acid (268), pregnancy (274), ovarian function (276), X-ray (11), parathyroid (108), parathyroidectomy and splenectomy (269), tetany and osteitis fibrosa (277), peptone shock (273), alcohol intoxication (266), and in pneumonia (275).

Reviews of the clinical conditions in which serum calcium is lowered or raised, or the distribution altered, have been given (7, 54, 117). Special studies have been made in pellagra (254) and in leprosy (264).

Calcium and phosphorus intake.—Subcutaneous and intravenous injections of calcium raised the serum calcium content temporarily (15, 66, 81); 25 mg. per kg. caused a maximum in one hour (with a return to normal in six hours) in normal and parathyroidectomized dogs (138). Calcium injections increased the non-diffusible calcium also (227, 249).

At various levels and ratios of Ca/P in the diet of rats, Kramer & Howland (131) found conditions which gave the same values of calcium and phosphate in the serum with and without vitamin D. and thus estimated the optimal intakes (cf. 29, 47).

Phosphates, by mouth or intravenously, lower serum calcium, as is well known (15, 64). Insulin, which lowers serum phosphorus, may or may not raise serum calcium (61, 64, 190).

Vitamin D.—The serum calcium and phosphorus reflect the Ca/P ratio of the intake when no vitamin D is present in the diet, and, when disproportionate, rickets, osteoporosis, osteomalacia, or tetany result. These conditions are cured, and the blood calcium and phos-

phorus tend to return to normal, when vitamin D is given [cf. Harris (89)].

Hypervitaminosis.—A flood of literature has appeared upon the effects of toxic doses of vitamin D (89, 90, 249). Unquestionably the blood calcium and phosphorus are raised, but to a different extent with various species, and with the same order of susceptibility as for parathyroid extract (247). In adult humans, 30 mg. of irradiated ergosterol (equivalent to 1.5 liters of average cod-liver oil) raised the serum calcium only 1 mg. per cent, with or without added calcium in the diet (26). Toxic doses of vitamin D produced a rise in serum calcium of dogs (120) and of rats (213) on a calcium-free diet, and of eviscerated cats (246). Taylor, Weld, Branon & Kay (247) hold that vitamin D stimulates the parathyroids, and after parathyroidectomy with complete dissection it is without effect on the relief of tetany. They, as well as others, after the usual operation, report alleviation of parathyroid tetany by large doses of vitamin D (16, 105, 120, 191, 212).

Parathyroid hormone.—The able and extensive review of Thomson & Collip (249) has covered the relation of parathyroid to the metabolism of calcium and phosphorus. The specific relief of parathyroid tetany and idiopathic hypoparathyroidism and the effects of parathyroid extract on raising blood calcium in normal animals are well known. Apparently, this same phenomenon occurs even after removal of the intestines (31). In parathyroidectomized animals, serum protein is not diminished, and the ratio of non-diffusible to diffusible calcium is not, surprisingly, increased above normal, but is probably diminished. The primary effect of parathyroid extract has been reported to be the fall of the blood-serum phosphorus, the rise in calcium being secondary (14). Measures which reduce phosphate, such as lactose administration, relieve tetany (155). A low phosphorus intake acts similarly (211).

Tetany.—Tetany is a condition of hyper-irritability of the neuromuscular system. It has been shown that small doses of curare abolish parathyroid tetany, which suggests that the condition of increased excitability lies in the nerves (96). Low blood calcium is usually associated with tetany, and may follow low calcium intakes (224). In all of the many forms except guanidine and magnesium tetany the symptoms may possibly depend upon diminution of ionized serum calcium. The diffusible (84) or adsorbable calcium (30) has been suggested as a better measure of tetany than the total. Even in

the absence of alkalosis, acidifying measures cause improvement (65, 164), and increased calcium intake is beneficial. Two new forms of tetany with low serum calcium have been described: (*a*) an idiopathic form in rachitic rats (220); (*b*) that which may follow hypercalcemia upon withdrawal of parathyroid administration (40, 112), or after operation in Recklinghausen's disease (cf. also magnesium tetany, p. 220).

A diminished serum phosphate has been reported in hyperventilation tetany (111). Rominger, Meyer & Bomskov (200) have reviewed spasmophilia, and have also given their theory that the cause of tetany lies in the phosphate retention of healing rickets—practically a phosphate tetany. It has been reported that the capillary blood is more acid than the venous blood from the longitudinal sinus—a central acidosis (48). The lowering of serum calcium caused by intravenous injections of glycine may be related to tetany (165). (Cf. 7, 54, 86, 249.)

CEREBROSPINAL FLUID

Cerebrospinal fluid normally contains about half as much calcium as the serum and about 38 per cent as much inorganic phosphorus. The fluid calcium varies with the level of serum protein (156). Injection of calcium salts increases the serum calcium more than that of the cerebrospinal fluid in normal and parathyroidectomized animals. Interesting studies on the dialysis of blood serum against cerebrospinal fluid in these two conditions showed the slight effect of parathyroid (162). After drainage of the cerebrospinal fluid, and during water excess with inhibition of diuresis, the calcium in the liquor remained a constant fraction of that found in the serum (156). In nephritis, the calcium of cerebrospinal fluid agreed with that of the ultrafiltrate only when serum calcium was normal (259). On this basis, the thesis that cerebrospinal fluid represents the diffusible calcium of serum seems untenable.

Studies were made also in pulmonary tuberculosis, in suppurative and nonsuppurative diseases of the central nervous system (156), in uremia (55), in epilepsy (122), and in the psychoses (201).

The aqueous humor of the eye of cats contains 6.0 mg. per cent calcium (157, 190); and amniotic fluid with a protein content of only one-thirtieth that of serum contains two-thirds as much calcium and one-third as much phosphorus (146, 157). Ascitic fluid has been compared to the dialysate of blood serum (78).

METABOLISM

Dietary requirements.—Sherman (217), who, perhaps more than anyone since Bunge, has emphasized the necessity for adequate intake of calcium in human dietsaries, again draws the distinction between maintenance and optimal intake, and recommends 0.75–1.0 gm. per day, especially in the diet of children. If the calcium intake is adequate, the phosphorus is usually sufficient. As a measure of adequacy, studies of balances, growth, longevity, pregnancy and lactation, and bone have been used. When the proportion of calcium to phosphorus in the diet is unbalanced, and vitamin D or ultra-violet light is absent, bone defects result (cf. also p. 215).

Growth.—Diets low in calcium but normal in phosphorus cause increases in body calcium proportional to the intake (219). In a study of the phosphorus deficiency of the rachitogenic diet plus vitamin D, retardation of body growth and bone growth was produced (221). In the absence of vitamin D, better growth was obtained with high-calcium, low-phosphorus diets than when the ratios were reversed; a Ca/P ratio of 1.0–2.0 gave the best results (29). Vitamin D improved growth, but did not obscure the effect of the Ca/P ratios and levels of intake (37, 106, 218). It has been claimed that the degree of rickets bears no relation to growth (20, 47); contra (196).

Absorption and excretion.—Nearly all forms of calcium, whether in milk or vegetables or salts, seem utilizable—even insoluble salts; tertiary calcium phosphate less than the primary and secondary (35). Phytin was absorbed (34, 67). Crude fiber did not impair absorption (36); contra (17). The favorable effect on calcium absorption of lactose (192) was not produced by glucose (94). Conversely, CaCl_2 , 0.02 per cent, increased absorption, and 0.32 per cent diminished absorption, of glucose from the isolated intestine of rabbits (144, 145).

Calcium is excreted principally by the intestine; in infants from 90 to 95 per cent, and in adults 60 per cent. Phosphate by mouth or by injection, in inorganic form or as glycerophosphate, caused increased excretion of calcium by the bowel (43, 62). Acid increased the urinary excretion of calcium and phosphorus; but alkaline diets did not increase retention (63, 206). Thyroid therapy increased excretion of calcium in both feces and urine without raising low serum calcium (18); similar results were obtained after tartrates and

citrates (82, 187); but acid, in a case with low serum calcium, paradoxically, increased only the fecal calcium (18). The elimination of calcium in the feces was diminished after administration of bile acids (70). Sodium acetate made the urine alkaline, but calcium acetate did not, because it was excreted by the bowel (263).

BALANCE STUDIES

The normal adult, in health, should approach a state of equilibrium with respect to calcium. Although in pregnancy positive balances are maintained, lactation is usually accompanied by loss of calcium from the body, sometimes with phosphorus retention. This depletion may continue for a year after cessation of lactation (58, 74, 141, 222, 252). Macy and associates were not able to report consistent storage when cod-liver oil and yeast were given. Steenbock, Hart, *et al.* (238), in their study of the lactating goat, which showed negative calcium balances when treated with ultra-violet light, state: "Antirachitic activation, therefore, cannot be considered a panacea for a disturbed calcium balance."

In infants, retentions were greater with a daily intake of a quart of milk than with a pint (172, 244). Roughage diminished balances in infants (60, 208). The minimum maintenance diets for growing children have been evaluated (256).

An interesting example of retentions in rapid growth of children (recovery from disease) has been given (235). In retention, a ratio of Ca/P lower than 1.5 indicated rapid tissue growth; one higher than 2.1 occurred only to replace a previous calcium shortage (232). That the ratio in retention is proportional to the intake, and is not markedly influenced by vitamins, has been re-emphasized: in lactating women (142); in heifers (140); and in rats (221).

In two normal men, potassium citrate was without effect on calcium, phosphorus, or magnesium retention (23) (in a nephritic it led to excretion of sodium and chlorine), and CaCl_2 led to temporary positive balances. In two women, high- and low-fat diets exerted no effect on calcium balances (149); contra (44). Retentions of calcium and phosphorus in humans were better with wheat than with oatmeal (53); contra for rats (237) and for dogs (225). On an exclusive meat diet two men showed negative calcium balances. The calcium in the urine was three times that in the feces, and the proportion of phosphorus even greater (153). Good utilization was found with

milk (135), and with milk and ice cream (129, 186); with cheese (148); and with soy beans (12).

Vitamin D.—Vitamin D increased retentions in infants with both cow's-milk and human-milk diets (208, 244). For the growing child, irradiation ameliorated calcium utilization on a low intake (59). In adults, on either high or low calcium intakes, doses of irradiated ergosterol as large as 5.0–30.0 mg., though they did not definitely alter balances, caused a slight shift of calcium and phosphorus from the feces to the urine (26, 107, 126). Taylor & Weld (246), as well as Harris (89), think that therapeutic doses of vitamin D increase absorption of calcium and phosphorus, in contradistinction to toxic doses, which cause resorption of bone and prevent excretion of calcium by the gut (230).

In a case of osteoporosis, due to inadequate calcium intake, high calcium intake alone (19, 24, 49) improved balances of both calcium and phosphorus, and these were enhanced by small doses of irradiated ergosterol (24).

Rickets has been divided into four phases by Rominger *et al.* (197, 199). In the first two, the phosphorus retentions are smaller than the calcium; in the last two, increased phosphorus retentions result in healing and tetany (200). They have applied their studies on thallium rickets in rats to human infants (198). It has been reported that, after administration of vitamin D, calcium and phosphorus retentions were parallel (68, 174).

Irradiated ergosterol acted as a specific in a case of fatty diarrhea with low blood calcium and tetany (sprue). Although lipolysis was diminished, reduction of fat intake did not result in improved mineral balances (24, 71, 185). Even when the bile was excluded from the intestine, vitamin D converted calcium and phosphorus balances from negative to positive (76).

Parathyroid.—The signs and symptoms of parathyroid tetany disappeared, and there was an increase in calcium balance and a slight decrease in phosphorus balance, when irradiated ergosterol was administered (27). The beneficial effects of thyroid medication upon the metabolism and course of hypoparathyroid disease were clearly demonstrated (18).

In hyperparathyroidism, a high urinary phosphorus and a low serum phosphorus are present. Phosphate ingestion caused a large excretion of phosphorus in urine and decreased urinary calcium (14, 52). Vitamin D did not ameliorate the condition (119).

Calcium balances increased after parathyroidectomy and diminished with excess parathyroid extract, and high calcium intake intensified the condition (51). Soós (231) concludes that, in rachitic rats, vitamin D increases the balance of calcium, but not blood calcium; and parathyroid increases the blood calcium, but not the calcium balance—the action of the two must be different.

Other diseases of mineral metabolism and bone.—These have been reviewed (117). Calcium and phosphorus metabolism were found to be normal in healing fractures (57), and in several bone and metabolic diseases (19). In calcinosis universalis, calcium was retained in excess on a high-calcium diet; metabolism of other minerals was not disturbed (25, 69). Two children with late rickets showed ample calcium and phosphorus balances, with consistently low serum phosphate (233, 236). In Paget's disease, positive calcium and negative phosphorus balances were reported (133, 134), or mineral metabolism was normal except for sulfur (188).

BONE

Composition.—The ash of tibias of normal newborn infants has been found to be invariant in percentage of calcium, regardless of the maternal calcium metabolism (42). With a wide variety of diets, the Ca/P of the bone of chicks and rats fluctuated narrowly around 2.15 (110, 221, 261). [Only in fluorosis was the Ca/P of the bone lowered, because the calcium content was diminished (154).] Therefore, the ash content of the fat-free bones has practically replaced the separate determination of calcium and phosphorus. The shafts and metaphyses have been analyzed separately (101). Quantitative studies of calcification in experimental rickets by ash percentage and X-ray pictures (protective) (116), and by the line test (curative) (32), have been made. The histological examination of the undecalcified bone (130) remains the court of last resort for the detection of the nature of the bone defect and the earliest stages of calcification. The trabeculae are the site of calcium storage in the bone (24a, 85, 163, 245).

For further treatment of calcium and phosphorus in bone, cf. Kay (124).

Calcification in vitro.—Studies by Robison & Soames (193) on phosphatase have continued. They recognize other obscure local factors besides the action of phosphatase on phosphate esters. For summary, see Kay (125). The importance of carbohydrate decompo-

sition products other than lactic acid has been emphasized (158). It has been reported that acidotic bone caused precipitation of CaCO_3 ; alkalotic bone, $\text{Ca}_3(\text{PO}_4)_2$ (150).

Pathological calcification.—This is the subject of a review by Barr (22). Calcification in skin (151) and calcinosis generalis (25, 69) have been studied. Steinitz (240) has issued a comprehensive review on calcinosis, and Meyer zu Hörste (159) on calcification of tissues. It is well known to result from excess of irradiated ergosterol or parathyroid extract.

FACTORS IN CALCIFICATION

a) *Intake.*—Normal concentrations of calcium and phosphorus in serum do not indicate whether the flow of minerals is to or from the bones: the ultimate source lies in the diet. In osteoporosis, due to an insufficient intake, normal blood values were found, with rarefied bones (24, 95). A low phosphorus intake resulted in "aphosphorosis" (147, 243, 248). The antirachitic effects of various forms of phosphate have been quantitatively studied; glycerophosphates were more potent than CaHPO_4 ; neither hypophosphites (136, 137, 211) nor metaphosphates (211) were effective.

b) *Ratio of Ca/P.*—Vitamin D corrects faulty intakes (216), and makes the utilization of the minerals more efficient, but it cannot fix the phosphorus in bone "if the phosphorus itself is not there to be fixed" (248). (See also blood, p. 208.) The absolute amounts of calcium and phosphorus ingested, and their relative proportions, are interdependent. The best levels and ratios with and without vitamin D have been given: for the rat (29, 36, 47); for the chick (93, 261); and for cattle (92). That no mineral supplement, without vitamin D, would prevent rickets in chickens has been stated (253). Turkeys are more susceptible to rickets than chicks (209).

c) *Heavy metals.*—Studies with thallium (198), beryllium (46), magnesium and strontium (166, 167, 168, 194), have increased our understanding of the etiology of rickets. When lead replaced calcium in the rachitogenic diet, severe rickets developed in rats (214). Magnesium or calcium intensified the effect of lead, but phosphate prevented the toxic effects—and rickets. The interrelation in the diet can now be written, instead of the ratio Ca/P: heavy metals/P.

d) *Acid-base.*—That NH_4Cl may ameliorate or cure rickets in infants has been reported (164); contra for rats (195). Acid and alkaline diets did not affect the bones of rats (160). The customary

diets for production of rickets are alkaline, but a diet which produced mild rickets when acid caused no rickets when neutral or alkaline (220). Obviously, the acid-base nature of the diet is secondary to other factors.

e) *Phosphatase*.—The relation of phosphatase to calcification and bone diseases has been reviewed by Kay (125).

f) *Vitamin C*.—In scurvy, calcium and phosphorus did not deposit in bone until vitamin C was given (28, 205). Lemon juice aided calcification in rickets in dogs (225).

g) *Vitamin D*.—The review by Goldblatt of experimental rickets treats the material exhaustively (73; cf. also 86, 89, 226).

Deficiency of vitamin D and minerals may lead to fetal rickets (152), or to congenital osteoporosis (251). Irradiated ergosterol preserved the calcium and phosphorus of the skeleton of the mother (rat) and increased the size and the calcium and phosphorus content of the young (173); contra (127). Lactation, regardless of "the dietary income" in vitamins and minerals, causes "drafts on the skeletal bank" (248).

h) *Hypervitaminosis*.—The earlier reports that excess of vitamin D causes demineralization of the skeleton have been confirmed (139) and denied (121, 126). In any event, the thinning of the shaft and deposition at the epiphysis clearly indicate an altered bone metabolism. The production of osteitis fibrosa by overdoses of vitamin D has been reported (75).

i) *Parathyroid*.—The relationship of parathyroid to bone has been extensively reviewed by Thomson & Collip (249; cf. also 114, 117). Parathyroid hormone, which causes increased elimination, must draw calcium from the bones. By sublethal doses in guinea pigs (38), in dogs (39), in rats and dogs (119), and in rabbits (203), osteitis fibrosa—Recklinghausen's disease—was produced. The action on the bones of thyroid and parathyroid extracts singly and in combination has been studied (183).

j) *Parathyroid, vitamin D, and Ca/P*.—Excess parathyroid may produce a condition similar to severe rickets which vitamin D prevents (210). The calcification of bone produced by vitamin D is prevented by parathyroid, but the pathological calcification is increased (108); contra (33). Parathyroid hypertrophy in rickets was shown by Erdheim. The thesis has been advanced that absence of vitamin D causes hyperfunction of the parathyroids, which in turn causes rickets (87, 88). However, parathyroidectomized rats have

been made rachitic and cured by irradiated ergosterol (181). Further, it has been shown that the effect of parathyroid hormone is proportional to the vitamin-D intake (161). The reverse of this condition, the effect of vitamin D on the relief of parathyroid deficiency, has been the subject of many studies, discussed previously.

The problem of the relation of the parathyroids and vitamin D to each other is further complicated by the fact that the actions of both are related to the calcium and phosphorus in the diet (51, 91, 118, 215, 257, 258). Shelling (211) found that in parathyroidectomized rats high-calcium low-phosphorus diets prevented tetany (161) and high-phosphorus diets caused it (with high or low calcium). The relation of the hormone has been shown, not only to calcium and phosphorus content, but also to the acid-base value of the diet (161).

The viewpoint of Greenwald has been re-emphasized, that the primary effect of parathyroidectomy is upon the phosphorus metabolism, and that retention of phosphorus causes depression of serum calcium (61, 211). Similarly, in rickets, alterations of calcium metabolism may be considered as secondary to those of phosphorus. Perhaps for the word "calcification" we should substitute "phosphorification."

k) Other glands.—The relationship of the endocrine glands, thyroid, spleen, and ovary to bones has been reviewed by Thomson & Collip (250). Anterior pituitary caused a fall in blood calcium (109). Extracts of lymph glands have been prepared which lowered the calcium or the phosphate of the blood, the calcium and phosphorus balances, the basal metabolic rate, and affected the phosphatase (175, 176, 177, 178). These findings have been confirmed in the same laboratory (128). If others can substantiate this thesis, and prove that the bone lesions are identical with those of rickets, the etiology of rickets will be further complicated—and perhaps clarified.

Although calcium, phosphate, and bicarbonate ions probably govern the precipitation of salts in bone, there is no means of determining them. Various authors have suggested theories of altered excretory thresholds, without being able to express the mechanism. The local factor in the bones, the phosphatase, the phosphorus compounds of the blood, the acid-base equilibrium, the vitamin D, parathyroid and thyroid hormones, and other factors are known to be simultaneously operative. To the reviewer the possibility of adequate explanation seems to lie in the future, and the utility of many of the

current theories is in stimulating the search for new data. It seems best, then, to describe the material frankly as fragmentary, and often conflicting.

MAGNESIUM

ANATOMY

Little has been written on the content of magnesium in the body (2, 3, 4, 5, 6, 7, 8, 9, 86). A diet of polished rice lowered the magnesium content of pigeons (also potassium, iron, and sodium) (298). The carcasses and leg bones of rats which received ultra-violet light showed only one-half as much magnesium as those unirradiated (297). The diminution of the magnesium content of rachitic rats was improved with phytin (34). The Ca/Mg ratio in brain and testicle has been correlated with age (283). The magnesium content of the bile affected the solubility of cholesterol (282), but increase in magnesium intake did not change the magnesium content of bile (284).

BLOOD SERUM AND CEREBROSPINAL FLUID

The magnesium content of blood or serum is normally from 1 to 3 mg. per cent. This value is practically constant under conditions which alter serum calcium. The ultrafiltrability of magnesium was found to be greater than that of calcium (259). That calcium- and magnesium-phosphate complexes are colloidal in nature was suggested as a result of ultrafiltration experiments (207).

After magnesium injections, serum concentrations of 5.6 and 20 mg. per cent were associated with depression and anesthesia, respectively (299, 304). Intravenous KCl relieved magnesium narcosis, presumably by raising blood calcium (271). Magnesium was the only mineral found in increased concentration in serum in essential hypertension (303). No alteration was reported in the psychoses of children (289). Magnesium tended to be low in pregnancy (259).

The magnesium of serum was diminished in magnesium deprivation (292), in rickets and thallium rickets (280), and in hypervitaminosis-D (280); in tetany it was raised, parallel to the phosphorus (290). Serum magnesium of cows was increased in puerperal paralysis, and lowered in "grass tetany" (223, 302). Parathormone caused an immediate transient rise in the serum magnesium of dogs, fol-

lowed by subnormal values (287, 301). This rise was accompanied by a fall in phosphorus,¹ and preceded the rise in calcium (61). Hypophyseal injections produced no effect on serum magnesium (109).

The magnesium content of cerebrospinal fluid is usually higher than that of serum, the ultrafiltrable lower, and therefore cannot represent the ultrafiltrate of the serum (259, 304).

METABOLISM

The outstanding study of magnesium is that of McCollum and associates (291, 292, 296, 300), who have convincingly demonstrated in rats and dogs that magnesium is essential to life. When the magnesium was reduced to 1.8 mg. per kg. of the diet, a striking chain of symptoms ensued—vasodilation, acceleration of the heart beat, and three or four series of convulsions ending in death. The magnesium content of the blood was reduced to one-tenth of its normal value. This syndrome necessitates a new physiological term—"low-magnesium tetany." The blood calcium remained normal, and hence the theory of the pathogenesis of tetany must be enlarged to conform to the old concept of irritability of Loeb, Howell, Mathews, and Meltzer.

The limits of intake for optimal growth have been determined (286, 293). Diets high in magnesium and low in calcium produced renal calculi (305). The relation of vitamin A and mineral deficiency to stone has been discussed (295).

The magnesium salts are easily absorbed; the soluble salts more readily. Irritants increased the absorption in dog and man (304). Approximately from one-fifth to one-half of the magnesium salts are excreted by way of the urine in infants and adults; the major portion, along with the calcium and phosphorus, in the feces.

The addition of magnesium to a diet rachitogenic for rats had an antirachitic effect (285); contra (166, 214). When magnesium was added to varying Ca/P intakes, the calcium and phosphorus balances demonstrated the interrelation of the three factors (278, 281). The balances of magnesium (also calcium and phosphorus) have been given for an infant (244), for children (288), for a man on a milk diet (135), for normals and a nephritic (23). No important

¹ Dr. L. Kajdi, in a pre-publication report.

changes were noted in calcium, phosphorus, or magnesium metabolism in polycythemia vera (279), or in Paget's disease (188).

Thus, magnesium metabolism should be considered as intimately related to that of calcium and phosphorus. Although these three, in certain aspects, function as a unit, a more accurate understanding occurs when the total mineral metabolism is considered.

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THE HORMONES*

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Limitations of space have again compelled us to restrict this review to certain special topics, especially to those in which, in our opinion, the most significant advances have been made; and we can but offer a general apology to those workers whose publications we have regretfully excluded from the discussion.

Oestrin.—Butenandt & Störmer have found that the distillation of trihydroxy-oestrin ($C_{18}H_{24}O_5$) from acid potassium sulphate yields two isomeric ketohydroxy-oestrins ($C_{18}H_{22}O_2$), of which the α -form is identical with that obtainable from human pregnancy urine ("theelin" of Doisy), while the β -form, which is only about one-sixth as potent physiologically, is said to occur in the urine of pregnant mares. Mares' urine apparently contains a remarkable mixture of different oestrins, for Schwenk & Hildebrandt obtained from it another isomer, the δ -form, said to be of rather high activity, and Girard and his colleagues had previously isolated two substances of lower potency which they termed "equiline" and "hippuline" and believed to have the composition $C_{18}H_{20}O_2$, though the difficulty of obtaining accurate combustion figures with substances of this type is notorious.¹ Schwenk & Hildebrandt were unable to obtain an oxime from their δ -form, yet at present we may probably consider all these six substances to be ketohydroxy-oestrins, whether isomeric or not; their characteristics are set forth in Table I (p. 232), and appear to show that all six forms are quite distinct. Whether conversion of one form into another can explain the changes in potency of certain preparations observed by both Marrian and Dingemanse must remain an open question. Butenandt affirms that the oestrogenic substance in plants (palm-nut oil) has been identified as a ketohydroxy-oestrin; Schoeller & Goebel suggest that it may have a physiological rôle in plants as a stimulator of the development of buds, though it is not identical

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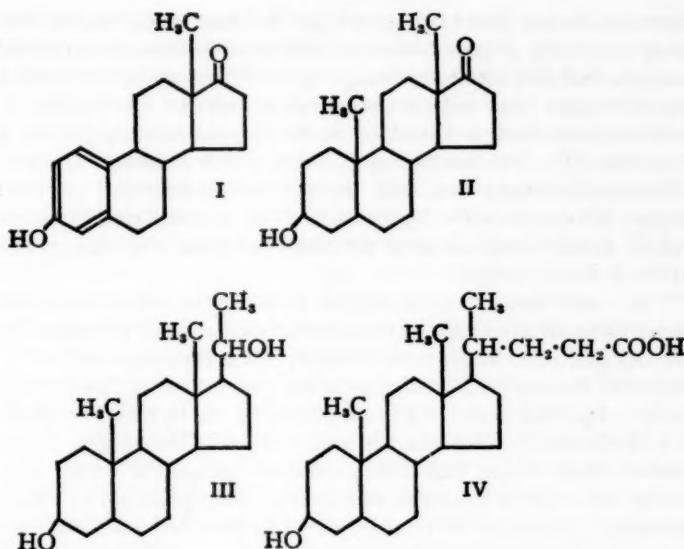
¹ In their latest paper, these workers describe yet another form, "equilene," of rather high solubility and marked acid properties; it is regarded as $C_{18}H_{18}O_2$, yet takes up only as much bromine (one atom) as the $C_{18}H_{22}O_2$ compounds, and may therefore contain a naphthalene nucleus; its physiological potency is low.

with the growth-promoting "auxin" which is responsible for geotropic and phototropic responses and which Kögl & Smit (cf. also Dolk & Thimann) have found in human urine and take to be an acid, $C_{18}H_{32}O_4$. Oestrogenic substances also occur in invertebrates and protozoa (Loewe *et al.*; Schwerdtfeder; E. E. Bauer) but physiological function has not been demonstrated in forms lower than teleosts

TABLE I
CONSTANTS OF KETOHYDROXY-OESTRINS

Substance	Rotation	Melting-Point	Benzoate m.p.	Oxime m.p.
α-form	$[\alpha]_D^{18}$	157° ($CHCl_3$)	255°	217.5° 230°?
β-form	$[\alpha]_D^{18}$	165° ($CHCl_3$)	257°	205°
δ-form	$[\alpha]_D^{22}$	46° ($CHCl_3$)	209°	177° None?
Equilin	$[\alpha]_D^{15}$	308° (dioxane)	239°	197° 222°
Hippulin	$[\alpha]_D^{15}$	128° (dioxane)	233°	
Equilenine	$[\alpha]_D^{16}$	87° (dioxane)	258°	222° 249°

(Fleischmann & Kann). In trihydroxy-oestrin, the two hydroxyl groups from which water is withdrawn in the formation of keto-hydroxy-oestrin are probably secondary and situated on a saturated five-membered ring, since Marrian & Haslewood (2), and MacCorquodale, Thayer & Doisy obtained by potash fusion a dibasic acid $C_{18}H_{22}O_8$ which yielded an anhydride; they consider that the compound has four rings, of which one is aromatic and the others saturated. Butenandt has also accepted this view, having obtained stable saturated compounds such as $C_{18}H_{27}(OH)_3$ by catalytic hydrogenation, and by distillation from zinc dust a hydrocarbon $C_{18}H_{14}$. Marrian & Haslewood (1) found that the acid properties of the oestrins were due to a phenolic hydroxyl, which is probably remote from the ketone group, according to the crystallographic analysis of Bernal. According to Butenandt, the male hormone is a saturated keto-hydroxy compound with four rings, probably $C_{19}H_{30}O_2$. If the assumption is made that keto-hydroxy-oestrin (I) and male hormone (II) are closely related to each other and—through pregnadiol (III)—to the sterols and bile acids, such as lithocholic acid (IV), tentative formulae may be set up as follows:



Butenandt & Störmer find that trihydroxy-oestrin is ordinarily contaminated with ketohydroxy-oestrin; when the latter is removed by semicarbazide, the activity falls to 75,000 mouse units per gram. MacCorquodale, Thayer & Doisy, however, were unable to find any ketohydroxy-oestrin in their preparation (Doisy's "theelol"). Collip, Browne & Thomson have shown that "emmnenin" from human placentae is an ether-insoluble complex from which autoclaving sets free in ether-soluble form a substance which they believe is identical with that which has been isolated from the ether-soluble fraction, and which has the chemical properties of trihydroxy-oestrin. In the adult castrate rat it is far less potent than Doisy's theelol and is comparable to Butenandt's pure trihydroxy-oestrin, but in the immature intact rat it is as active as theelol. They suggest that it undergoes conversion in the ovary to some more active form. The active principle of follicular fluid, according to Curtis, resembles ketohydroxy- rather than trihydroxy-oestrin. Cook, Dodds & Hewett made the striking discovery that 1-keto-1,2,3,4-tetrahydrophenanthrene has considerable oestrogenic activity.

Anterior pituitary.—A number of workers have now mastered the difficult technique of hypophysectomy in the rat, and a new field of

investigation has thus been opened up. Reichert *et al.* reported that pregnancy-urine preparations were ineffective in hypophysectomized animals, but Noguchi had already reported their ability to produce pseudocorpora lutea and the phenomena of oestrus. Comparable results were obtained by Freud, by Smith (2), and by Collip, Selye & Thomson (1). The last-named also found that treatment of hypophysectomized male rats with the anterior-pituitary-like placental extract did not check the degeneration of the germinal epithelium but caused marked development of the interstitial tissue with enlargement of the accessory organs.

It is well known that castration leads, in the rat, to increased ovary-stimulating potency of the anterior pituitary and to increase in the basophil cells, which have therefore often been regarded as the source of the ovary-stimulating principle (see, however, Kraus); but Severinghaus has shown that in the guinea-pig the increase in potency is accompanied by an increase in acidophil cells. Hohlweg & Dohrn reaffirm their finding that these castration changes, in female rats, can be prevented by treatment with oestrin. They point out that since castration changes in the pituitary occur in immature rats, even immature ovaries must secrete oestrin in amounts estimated at about one-twentieth of a unit daily. Reese & McQueen-Williams prevented the appearance of castration cells in the pituitary of male rats by injecting testicular hormone. McCullagh, however, ascribes this effect, not to the testicular hormone as ordinarily defined, but to a water-soluble principle. Martins & Rocha declared that ovarian grafts were active in this respect in female rats, but not in male rats; but Haterius & Nelson find them active in either sex.

Riddle, Bates & Dykshorn have obtained, by isoelectric precipitation of an acid extract of anterior pituitary, a fraction which they term "prolactin" and which stimulates the development of the crop-gland in male, female, or castrate pigeons, an effect not obtainable with gonad-stimulating or growth-promoting extracts nor with pregnancy-urine preparations. Prolactin is said also to cause secretion in the fully developed mammary gland in male and female guinea-pigs and in female rabbits. Corner's view that anterior pituitary extracts may lead to full development of the mammary gland in ovariectomized rabbits is confirmed by Turner & Gardner; in the rat, however, this stimulating extract is effective only in presence of the ovaries, according to Evans & Simpson, and Turner & Schultze, and our own experience with placental extracts. The positive effect obtained by

Ferrigno with pregnancy urine may not be wholly due to anterior-pituitary-like substances. Probably much depends on the stage to which the mammary glands have developed when treatment begins. Thus Bradbury finds in mice that if interlobular tissue is once formed (for example under the influence of secretions from ovaries stimulated by pituitary or pregnancy-urine preparations), then pituitary extract will induce formation of alveolar tissue even after ovariectomy, while pregnancy-urine preparations fail. The induction of actual secretion is another question again. Collip, Selye & Thomson (1) find that hypophysectomy, unlike ovariectomy, leads to a prompt cessation of lactation, and no means of restoring it has yet been discovered. It is conceivable, in the light of the experiments of Fauvet (2), that the posterior pituitary is concerned in this. The delayed effect of sympathectomy on lactation in the dog (Cannon & Bright) and rat (Bacq) may possibly involve the pituitary.

The effect of the anterior pituitary upon the thyroid gland has been much studied in the period under review. The administration of suitable preparations to guinea-pigs leads to vacuolisation and increased height of the acinar epithelium, eventually almost to obliteration of the lumina, to discharge of colloid, and to increase in thyroid weight. Dried pituitary powder has been used as such by Janssen & Loeser, saline suspensions by Schockaert, acid extracts by Loeb and his collaborators, pyridine extracts by Loeser (2), and alkaline extracts by Krogh, Lindberg & Okkels. Loeser (2) has described means of purifying the active principle, removing inactive proteins with trichloracetic acid, and Junkmann & Schoeller have also obtained highly potent preparations, apparently with sulphosalicylic acid; in contrast to most other workers, they emphasize the thermolability of the active principle. Similar effects have been obtained in the dog by Janssen & Loeser, and Grab (1), in the rabbit by Schittenhelm & Eisler (3), in the hen by Noether, and in the duck by Schockaert. The rat, however, appears to be resistant to this treatment, although its pituitary contains large amounts of the active principle (Loeb; Junkmann & Schoeller; cf. also Anderson).² That the histological changes really do indicate an increased outpouring of thyroid secretion is attested by many observations. Thus a reduction in the iodine

² Compare also Houssay; yet our recent experience has been that 100 guinea-pig units will produce definite hyperplasia in normal rat thyroids, and Schittenhelm & Eisler (3) have also obtained positive results; we have also been able to check thyroid atrophy in hypophysectomized rats.

content of the gland was observed in the dog by Loeser (1) and Grab (2), in the guinea-pig by Closs, Loeb & Mackay, and by Houssay, Mazzocco & Biasotti, and in the duck by Schockaert & Foster; while a rise in the alcohol-insoluble iodine of the blood has been seen in the guinea-pig by Closs, Loeb & Mackay, in the dog by Grab (2) and Schittenhelm & Eisler (3), and by the last-named also in man. The blood acquires the power to protect mice against acetonitrile, according to Grab (1) and Oehme, Paal & Kleine; an increase in heart rate was seen by Schittenhelm & Eisler (3) in man, and by Hageman & McCordock in the guinea-pig, in which species there may also be exophthalmos [Loeb & Friedman (2)], discharge of liver glycogen (Eitel & Loeser), increased metabolic rate (Siebert & Smith; Verzar & Wahl), and increased susceptibility to anoxemia (Houssay & Rietti). In many cases it has been possible to show that the effects are dependent on the presence of the thyroid, and they are independent of the presence of the gonads (Janssen & Loeser); in fact, when a pronounced thyroid response is obtained, ovarian activity is depressed, according to Noether, and Loeb & Friedman (1). It seems clear, too, that gonad-stimulating extracts may be obtained free of thyrotropic effect, and there is general agreement (Janssen & Loeser; Junkmann & Schoeller; Loeb; Schockaert & Foster; Verzar & Wahl) that pregnancy-urine preparations have no action on the thyroid. The opposite view is expressed by Caulaert, Aron & Stahl, but their assay technique has been criticized by Castillo & Magdalena, and others. Oehme, Paal & Kleine also regard the active principle as present in urine, but their account of it as quite stable to heat and active orally is also surprising, and their assays are conducted by direct or indirect Reid-Hunt (acetonitrile) tests in mice without reference to thyroid histology. The discharge of thyroid colloid which follows copulation in the rabbit (Krilov & Sternberg) may conceivably be due to a general discharge of anterior-pituitary hormones; it is apparently accompanied by a fall in serum calcium, which can also be produced by injection of pituitary suspension (Hogben & Charles), and of course there can be no doubt that the ovary-stimulating hormone is set free in large amounts.

Simon & Binder have confirmed Van Dyke's experiments with the anterior-pituitary growth hormone; but Van Dyke's extract ("phyone") has been found by Schockaert to have thyrotropic effects, and the claim that it is free of gonad-stimulating principles

must be abandoned, in the light of the work of Leonard, of Hain, and of Hertz, Hellbaum & Hisaw. Collip, Selye & Thomson (2) have described a highly purified growth-promoting extract. Wehefritz & Gierhake claim that they have detected the growth hormone in pregnancy urine.

Cushing (2) has described a clinical syndrome of adiposity of the face and trunk with dusky skin and *striae distensae* on the abdomen, with kyphosis, amenorrhoea or impotence, hypertension, and sometimes osteoporosis and hypertrichosis. He believes that this syndrome is due to hypersecretion by the basophil cells of the anterior pituitary, though the presence of basophil adenoma was not established in all cases, and the adrenal cortex was frequently hyperplastic. We do not feel that the cause of this syndrome is definitely established; it is not easy to conceive what can be the normal function of the hormone supposed to be present in excess in such cases.

Uhlmann, and Zondek & Bier, have rediscovered the relatively high bromine content of the anterior pituitary. On evidence which appears slender even if one admits the accuracy of the bromine determinations, the latter workers suppose that an organic bromine compound passes from the pituitary to the brain and produces sleep and sedative effects.

Houssay & Biasotti (1) found that in hypophysectomized toads pancreatectomy did not cause severe diabetes. Phlorhizin also causes less glycosuria than usual (Di Benedetto). Implants of anterior pituitary restore the normal reaction, and Houssay & Biasotti (3) have also prepared active extracts from the anterior lobes of fishes, batrachians, birds, and mammals; the active principle is thermolabile and precipitable by acetone. Houssay & Biasotti (2) also found that in hypophysectomized dogs pancreatectomy produced only mild diabetes, with low dextrose-nitrogen ratios and no rise in basal metabolic rate (thyroidectomy however does not lessen diabetes),³ while phlorhizin produces marked hypoglycemia with low dextrose-nitrogen ratios (Biasotti & Houssay) and little ketosis (Rietti). It is suggested that hypophysectomy checks the production of carbohydrate from endogenous protein, since it reduces the fasting excretion of creatinin (Braier). The injection of extracts of anterior pituitary

³ We feel nevertheless that the possible rôle of thyroid atrophy in these phenomena has not been sufficiently considered; Dann, Chambers & Lusk found that thyroidectomy lessened the severity of phlorhizin diabetes, and analogous observations have been made in depancreatized dogs.

increases the amount of acetone bodies in the blood, according to Anselmino & Hoffmann (2) and Magistris, and occasionally produces glycosuria (Evans, Meyer, Simpson & Reichert; Baumann & Marine).

Posterior pituitary.—There is little in the recent literature to dispel the doubts of the increasing number of workers who question whether the active substances of the posterior pituitary ever escape into the circulation and play a physiological rôle in the healthy mammalian organism. Friedman & Friedman could find no oxytocic substance in cerebrospinal fluid, using the rabbit uterus *in situ* as a test object. Ivy, Hartman & Koff studied the effect of pitocin on the monkey uterus at term, and did not feel that it could play any useful rôle in the birth mechanism. Allan & Wiles in the cat, and Smith (1) in the rat, concluded that the posterior lobe was not essential for parturition, and this view is supported by the observations of Pencharz & Long (who stress the importance of the anterior lobe) and by unpublished findings in our laboratory.

The histological evidence that secretion takes place into the third ventricle is dismissed by Bucy in a recent review; but an entirely new aspect of the question has been revealed by the work of Cushing (1), who finds that pituitrin injected into the ventricle, in human subjects, has effects very different from those seen after subcutaneous injection. The principal effects are persistent flushing of the skin and sweating (not observed in denervated areas), fall of body temperature and of blood pressure and pulse rate, lachrymation and salivation, and nausea. All these effects are prevented by atropine and by non-volatile anesthetics such as avertin, and are exactly similar to those produced by intraventricular pilocarpine. Histamine, however, has quite different properties. It is clear that those who wish to believe that posterior-pituitary principles pass into the ventricle must seek support for the theory in effects not hitherto considered in this connection. Yet it is still possible to believe that the phenomena which Cushing describes are due to some hitherto unknown and possibly non-specific constituent of pituitrin. Cushing (1) believes that pituitrin or pilocarpine, given intraventricularly, stimulates some "parasympathetic centre" in the hypothalamic region, from which impulses radiate to produce the observed effects. This suggestion is not altogether new. Thus Yamamoto finds that pituitrin has more effect in raising blood sugar when given subarachnoidally than when given subcutaneously, and that the effect is abolished by

spinal transection. Raab had previously treated the action on the blood fat from a similar standpoint, and recently Silbermann has found that pituitrin does not exert its usual antidiuretic effect in patients with transverse lesions of the cervical cord (although it undoubtedly does have some action on the isolated kidney under suitable experimental conditions). Lawrence & Dial investigated the effects of intraventricular injection of pituitrin in dogs, but owing to the inhibitory action of anesthetics and the difficulty of finding a controllable test-object, decisive results were not obtained. Later, however, Light & Bysshe obtained more definite results with unanesthetized *Cercocebus* monkeys. They found that intraventricular pituitrin or pitressin caused a marked flushing of the oral mucous membranes, which was succeeded by pallor when the drug entered the circulation. Pitocin, histamine, acetylcholine, and pilocarpine did not have this effect when given intraventricularly.

Anselmino & Hoffmann (1) believe that in eclampsia the blood contains pressor and antidiuretic substances of pituitary origin, and Dietel has confirmed the similar claim for the presence of the melanophore-expanding principle, while Fauvet (1) produced lesions of the liver and kidney of rats and guinea-pigs, similar to those of eclampsia, by injections of "hypophysin." Zondek & Krohn have found a new and more specific test-object for the melanophore-expanding principle in the erythrophores of the minnow, *Phoxinus laevis*. They name the principle "intermedin" because of its presence in highest concentration in the pars intermedia. Like Dietel, they believe it to be independent of the pressor and oxytocic principles. Hogben & Slome believe that the amphibian hypophysis also contains a substance which contracts the melanophores; such dual control has previously been postulated for crustacean colour change and recently also in fishes (Mills).

Geiling & DeLawder made an observation of fundamental importance when they discovered that the effect of intravenous injection of pitressin was to check oxygen uptake by the muscles from the blood, so that the venous blood becomes temporarily arterial in character, while blood lactic acid increases (Nitzescu & Munteanu; Himwich, Haynes & Fazikas) but there is apparently little change in glycogen distribution (Bischoff & Long; Lawrence & McCance; Hynd & Rotter), the technique used by Gömöri & Marsovszky to demonstrate a fall in liver glycogen being open to criticism. Grollman & Geiling found similarly that the injection of pituitrin in

unanesthetized human subjects led to a sharp fall and a delayed rise in oxygen consumption. After a transient initial fall the cardiac output and pulse rate increased, but in spite of this and the obvious cutaneous vasoconstriction the blood pressure fell. It is clear that the action of pituitrin or pitressin on arterial blood pressure is the algebraic sum of various local effects, which differ with species (Holtz) and anesthetic (Raginsky & Stehle). Holtz has shown that the fall in portal pressure (McMichael) and decrease in liver volume (Emery & Griffith) are due to the oxytocic fraction.

Adrenalin.—Annau, Huszak, Svirbely & Szent-Györgyi found that extracts of adrenal gland made rapidly at low temperature had ten to fifteen times more physiological potency than colorimetric estimation suggested. This may be compared with the observation of Mouriquand & Leulier that extracts of perfectly fresh glands give only a faint Deniges test. Meanwhile Kendall had found a compound of adrenalin and lactic acid in acetone extracts, and prepared a similar substance synthetically, but he apparently regards the physiological activity as small.

Tournade has found that when the kidney of a dog "A" is perfused by cross-circulation from dog "B", injection of adrenalin into "A" causes constriction of the renal blood-vessels which can only be due to the nervous connections and points to a central action of adrenalin. The interesting observations of Samson on the effects of prolonged infusion of adrenalin unfortunately do not lend themselves to summary discussion.

Thyroid.—A marked increase of confidence in methods for determining small amounts of iodine has led to a series of studies of the fate of thyroxin in the human body. Böe & Elmer find that blood iodine returns to normal a few hours after thyroxin injection, while the rate of excretion of iodine in the urine is increased only for the first day, and only a small part of the iodine supplied is recovered. Müller & Fellenberg obtained similar results. Schittenhelm & Eisler (1) studied fecal excretion as well. They found this to be considerable in normal or hyperthyroid individuals (receiving thyroxin orally) and relatively small in myxedematous patients; their total recoveries were large. But Thompson & McLellan do not find that the calorigenic action of thyroxin is enhanced by dividing the dose, which suggests that excretion is not rapid. The iodine of the feces is not merely a fraction which has not been absorbed, since Asimoff & Estrin found iodine and even thyroxin in the bile of treated

dogs. Boothby, Buckley & Wilhelmj studied the rate of decay of the calorigenic effect in dogs. They found it not to be increased by exercise.

Leland & Foster separate thyroxin from hydrolysates of thyroid protein by butyl alcohol extraction, and find one quarter of the total iodine of normal human glands in this fraction. Gutman, Benedict, Baxter & Palmer used this method to show that the amount of thyroxin is even more strikingly subnormal in exophthalmic goitre than the amount of total iodine, and that iodine therapy increases the amount of thyroxin not only absolutely but also, in slight measure, relatively to the total iodine. Similarly Krogh & Lindberg found that the biological activity per unit of iodine was low in glands from cases of exophthalmic goitre, indicating a low ratio of thyroxin-iodine to total iodine. Palmer & Leland obtained only inconclusive results in similar experiments.

The site of action of thyroxin remains a subject of discussion. Dock & Lewis find that all parts of the body share in the increased metabolism. The metabolism of tissues from thyroidectomized animals is low (Davis & Hastings; Hicks), but high from thyroid-treated animals (Hopping; Hicks; McEachern; Gerard & McIntyre); in general, however, it is impossible to affect the metabolism of isolated tissues by adding thyroxin *in vitro*. Verebely finds that brain tissue is an exception to this rule, but we need not conclude with him that this proves the action of thyroxin to be central, nor need we draw a similar conclusion from the finding of Schittenhelm & Eisler (2) that thyroid treatment increases, and thyroidectomy decreases, the iodine content of the hypothalamic region. Markowitz & Yater found that additions of thyroxin accelerate the rate of beat of explanted chick heart tissue, and Euler found that minute quantities of thyroxin promptly increased the oxygen consumption of the isolated perfused hind-limb. The effect of thyroid treatment is not impaired by spinal transection (Oberdisse) nor by sympathectomy (Ring, Dworkin & Bacq).

Haney stimulated the cervical sympathetic in rabbits for an hour and found the metabolic rate increased for several weeks thereafter. He concludes that he has found the secretory nerve to the gland, but it is surely possible that he had evoked a secretion of pituitary thyrotropic hormone, even if one can assume that infection (whose rôle in the production of thyroid hyperplasia has been pointed out by Mahorner and Anderson) was not present. Vogt found that de-

nervation of the thyroid did not prevent the usual histological responses to cold, starvation, etc. Rein, Liebermeister & Schneider find that blood flow through the gland varies reflexly with the pressure in the carotid sinus.

The thyroid hyperplasia produced in rabbits by a diet of cabbage (see review by Webster) is ascribed by Marine, Baumann, Spence & Cipra to the presence of mustard oils, since they were able to produce similar goitres, preventable by iodine, with acetonitrile and similar substances. McCarrison finds that the iodine content of the enlarged glands is low. Marine and his colleagues believe that hyper-activity of the thyroid is an attempt to compensate for decreased power of the cyanide-poisoned tissues to take up oxygen. It may be objected to this view that thyroid treatment is said not to confer protection against acetonitrile, except in mice. There appears to be considerable doubt as to what actually is happening. Thus Marine, Spence & Cipra describe signs of hyperthyroidism, such as exophthalmos, nervousness, and diarrhea, whereas Baumann, Kurland & Metzger find the mineral metabolism to be of hypothyroid type with retention of calcium.

Neither Hellwig nor Jackson & P'an could produce goitre in rats merely by reducing the iodine intake; but if the calcium intake is raised at the same time, goitre results (Hellwig; Levine & Remington; Thompson). The Steenbock rachitogenic diet (with or without added vitamin D) is a ration of this character, although Sekiguchi did not find any effect with it upon the thyroid. A vitamin-deficient diet also produces goitre with evidence of hypothyroidism in rats (Spence), and in rabbits (Sandberg & Holly), and exclusion of ultra-violet light has a similar effect both in rats (Nitschke and Bergfeld) and in chickens (Turner & Benedict), though there is not complete agreement as to the histological pictures and metabolic effects produced by such measures.

Parathyroids.—The literature up to the end of 1931 has been reviewed elsewhere (Thomson & Collip), but several important observations have been made since then. Allardyce re-investigated the optimal conditions for the preparation of active extracts. He was unable to find any new principle in purification, but Tweedy & Smullen precipitated an active fraction from phenol with ether. Tweedy & Torigoe found that activity was abolished reversibly by treatment with formaldehyde or with acid alcohol, and irreversibly by nitrous acid or acetic anhydride. These results are most simply explained

by supposing that the "active group" of the molecule carries a primary amino group.

The mechanism by which hypercalcemia is produced has been discussed from several points of view. Albright and his collaborators, especially Ellsworth, believe it to be secondary to a fall in plasma inorganic phosphate, and propose to treat hyperparathyroidism by phosphate administration. But Grauer found in the guinea-pig that phosphate enhanced the destructive action of parathyroid extracts on bone, and Thomson & Pugsley were unable to find any fall in plasma phosphate in the dog, and when they produced such a fall by administration of glucose and insulin they saw only trivial increase in serum calcium. They also brought forward evidence against the theory that the parathyroid hormone forms a soluble complex with calcium. Greenberg and Greenberg have destructively criticized the evidence that any diffusible organic calcium complex is present in serum. The histological studies of Jaffé, Bodansky & Blair, of Büllbring, and of Selye (1) support the view that the hormone acts directly on the bones, stimulating osteoclasia. Selye (1) believes, in fact, that there is a generalized action on susceptible connective tissue, since he has been able to produce a condition resembling scleroderma in suckling rats. The rise in serum magnesium observed by Scholtz and by Greenberg & Mackey is of great interest in that it is one of the earliest observable effects of parathyroid administration. Important, too, is the observation by Greenberg, and by Nitzescu, that injury to the liver prevents response to parathyroid extracts.⁴ Gürsching & Kraut affirm that the silica content of the blood varies along with the serum calcium.

Bryan and Garrey have made an illuminating analysis of the rôle of rising temperature and hyperventilation in the production of parathyroid tetany. Beznak (1) ascribes the observed rise in plasma

⁴ It seems to be entirely possible that the bone cells were injured by the phosphorus used to produce liver damage. An important contribution to the theory of parathyroid action has been made by Hastings & Huggins, who found that blood could be decalcified by shaking with solid lead phosphate and could then be reinjected; but in normal dogs, or six hours after parathyroidectomy, the restoration of serum calcium is astoundingly rapid, whereas 24 hours after parathyroidectomy it is slow. This suggests that healthy osteoclasts are stimulated by hypocalcemia to liberate calcium, but that they lose this responsiveness when the supply of parathyroid hormone is withdrawn; other interpretations are of course possible.

inorganic phosphate partly to breakdown of phosphocreatine, and Imrie & Jenkinson believe that thyroparathyroidectomized cats have great difficulty in resynthesizing phosphocreatine in muscle. Conversely, Brown & Imrie find that the simultaneous administration of parathyroid extract and creatine checks the excretion of phosphate in normal cats. This does not agree with the view of Albright that parathyroid treatment lowers the renal threshold for phosphates. Bülbbring has carried out an extensive investigation of the effect of parathyroidectomy and parathyroid administration on the calcium and phosphorus balances and the composition of the tissues and the bones in rats on diets of varying calcium content. She found that parathyroidectomy tends to increase the amount of calcium and phosphorus in the body, while parathyroid treatment decreases it, especially on diets of high calcium content. Pugsley also found that the daily administration of parathyroid extracts increased the excretion of calcium and phosphorus in rats, the most striking feature being the enormous increase in urine calcium, which, however, persists only a few days, after which the rats appear to be immune. Dyer noted similar increases after single doses, and suggested that the effect offered a means of assaying the active principle, which Hamilton & Schwartz propose to do by following the serum-calcium time-curve after oral administration of calcium chloride in rabbits, the degree and duration of the hypercalcemia being enhanced by small amounts of parathyroid extract. The "immunity" noted by Pugsley and others has been explained by Selye (2), who finds that the continued administration of parathyroid extracts in rats leads at first to increase in osteoclasts and rarefaction of bone, but later to increase of osteoblasts and bone apposition, eventually even to a picture resembling Albers-Schönberg "marble-bone" disease. This observation may be correlated with the finding of Bodansky & Jaffé, and of Holtz, Isemer & Stichnoth, that hypocalcemia follows prolonged parathyroid treatment in dogs.

Taylor, Weld, Branon & Kay believe that the action of massive doses of irradiated ergosterol is to stimulate the parathyroids. While they agree with a host of other workers that irradiated ergosterol may protect animals against tetany after ordinary parathyroidectomy, they claim that if extensive dissection is carried out so that all accessory parathyroid tissue is removed, the effect is no longer obtainable. Dale, Marble & Marks failed to confirm this claim, which has also been criticized by Shelling. Taylor and his collaborators believe also

that the pathological picture of overdosage with irradiated ergosterol is identical with that of parathyroid overdosage, but differences have been pointed out by Brand, Holtz & Putschar, by Pincherle & Ortolani, and by Selye (1). King & Hall observe that irradiated ergosterol is toxic in the chicken, whereas parathyroid extracts are not, though they agree with Macowan and Parhon & Parhon-Stefanescu that the extracts do have some action upon the bones.

Robinson & Thompson affirm that the parathyroids contain, as well as the substance active in producing hypercalcemia, a substance which inhibits growth. In our opinion the specificity of this effect is not yet convincingly demonstrated.

Acetylcholine and depressor bases.—The original experiments of Loewi, demonstrating the liberation of a substance resembling acetylcholine on stimulation of the cardiac vagus, have been confirmed by Hirschberg. More interesting is the extension of this idea to the mammal. Dale and Gaddum studied closely the contractures produced by stimulation of the chorda-lingual nerve in the dog, after degenerative section of the hypoglossal, and concluded that they were most readily explained on the theory that acetylcholine was liberated. Subsequently Bain perfused the dog's tongue *in situ* and found that the perfusate, collected during lingual stimulation, could stimulate isolated rabbit intestine. Rasenkov & Ptchelina concluded, from cross-circulation experiments, that a secretagogue substance was liberated when gastric or pancreatic secretion was evoked by vagal stimulation, but Freeman, Phillips & Cannon could find no sign of a circulating parasympathomimetic substance after stimulation of the vagus trunk, even in the presence of physostigmine (eserine), which protects acetylcholine from the esterase in blood (Engelhart & Loewi; Matthes). Babkin, Gibbs & Wolff found that stimulation of the chorda tympani in the physostigminized cat led to a fall in blood pressure not wholly due to local vasodilatation. Gibbs and Szelöczey devised means of intermittently perfusing the submaxillary gland, and found in the perfusate, during chorda stimulation, a substance which resembled acetylcholine in its action on the isolated frog heart (cf. Henderson & Roepke), on isolated rabbit intestine, and on the blood pressure of the cat, and which is destroyed by blood except in presence of physostigmine. Babkin, Alley & Stavraky found that stimulation of the chorda on one side increased secretion from the denervated submaxillary gland of the other side, and Beznak (2) obtained similar effects in cross-circulation experiments on dogs. Plattner and

Bischoff, Grab & Kapfhammer, have found relatively enormous amounts of acetylcholine in blood, muscle, and other tissues. The demonstration appears to be convincing, though Dale was unable to confirm it, yet it must be supposed that the acetylcholine is in some way combined or inhibited so that its pharmacological properties are masked.

The list of depressor constituents of tissues continues to grow. In addition to choline and its esters, histamine, adenosine and adenylic acid—perhaps also cytidylic acid (Drury)—we have to reckon with kallikrein (padutin). This substance, according to Frey, Kraut & Schultz, is formed in the pancreas, is reversibly inactivated by blood plasma, but reappears in active form in the urine. Preparations from this source have been purified (Frey), and are distinguished by thermolability and inability to dialyse, as well as by the action of blood. A. Bauer, however, failed to detect any such substance in blood or urine, and identifies the chief depressor substance of urine with the unknown alkali-unstable dialysable tissue constituent discussed by Euler & Gaddum and by Lange. This substance is distinguished from the choline group in being unaffected by atropine, from histamine in being depressor in the etherized rabbit, and from adenylic acid in being excitatory to rabbit intestine and in not producing bradycardia. Felix & Putzer-Reybegg found it in the arginine fraction in Kossel-Kutscher analysis. But the depressor activity of brain extracts was found by Weber, Nanninga & Major in the lysine fraction, and was stable to alkali; yet it may be premature to conclude that they are dealing with a different substance. Christie has found an unidentified depressor substance in large amounts in a human carotid-body tumor and, along with adrenaline, in the carotid bodies of elasmobranchs.

Very interesting is the evidence which points to liberation of histamine in anaphylactic shock. Bartosch, Feldberg & Nagel found that when an antigen was added to the fluid with which the lungs of a sensitized guinea-pig were perfused, the perfusate in turn could produce spasm in the lungs of a normal guinea-pig. Watanabe had shown that the histamine content of the lung decreased rapidly during shock, and Dragstedt & Gebauer-Fuelnegg found a histamine-like substance in the thoracic lymph of the dog in anaphylactic shock. Sacks, Ivy, Burgess & Vandolah have definitely identified the secretagogue substance of gastric mucosa as histamine.

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VITAMINS*

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Activity in this field has continued intense, and a number of striking advances have been made. The rising flood of literature shows no sign of abating. The following figures give the number of papers which became available during the period under review (late 1931 to late 1932): vitamin B₁, 168; vitamin B₂, 56; alleged new B vitamins, 18; bios, factors for micro-organisms, etc., 17; vitamin C, 127; vitamin A, 221; vitamin D, 332; vitamin E, 19. Thus the total falls little short of the 1,000 mark, or roughly three new papers appear each day. It had been hoped to present here a comprehensive synopsis of the year's work, but the necessarily limited amount of space available has forced the omission of considerable portions of our review. In particular, little reference will be found to the following aspects: clinical studies on the avitaminoses; anatomical and histopathological investigations, and many metabolic measurements, mostly giving negative or confirmatory results; determinations of vitamin distribution in numerous classes of foodstuffs—unless the results were specially noteworthy; variations in methods of vitamin assay; and much purely confirmatory work supporting results already well established (e.g., on hypervitaminosis).

PART I. WATER-SOLUBLE GROUP

VITAMIN B₁ (B)

Physiology of vitamin-B₁ action.—Many papers published during the year strengthen the belief that vitamin B₁ is concerned in carbohydrate metabolism. Information is now being won concerning the stage at which it intervenes.

It will be recalled that the connection between carbohydrate metabolism and vitamin-B action first suggested itself many years ago to Funk, who showed that polyneuritic symptoms in birds were aggravated by a diet rich in carbohydrate. Similar studies on birds recently published confirm the conclusion that glucose tolerance, as measured by the blood-sugar curve (Nitzescu & Benetato), is less in

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avitaminous than in normal birds, and show that the absence of carbohydrate from the diet prolongs the life of the polyneuritic birds (Yanovskaya, Lecoq). (Studies have been made also of the varying effect of different carbohydrates: Randoin & Lecoq.) With the gradual decrease in tolerance for carbohydrate, there occurs an increase in the blood sugar and a rise in the lactic-acid concentration in the blood and elsewhere, lactic acid being excreted in increasing amounts in the urine. This state of abnormal metabolism is confirmed and well illustrated by recent observations on dogs (Kauffmann-Cosla, Vasilco & Oerul; Braier). Vitamin B, then, seems to have the effect of indirectly reducing the concentration of the blood sugar (current work by Lelesz & Przeździecka illustrates this) and also of removing lactic acid such as that which accumulates during exercise. In keeping with the latter effect is the finding that the slow heart rate, observed in vitamin-B deficiency by Drury and the writer, appears to be due to the lactic-acid excess (Birch), and the finding of Miyami that vitamin B has a beneficial effect in promoting recovery from fatigue, as measured by the electrocardiogram.

Peters and his co-workers have continued to study the error in lactic-acid and glucose metabolism in relation to the brain tissues. In earlier work they had attributed the convulsions in vitamin-B deficiency to this defect in the brain. Two papers published recently deal with *in vitro* experiments. In the first (Gavrilescu & Peters) they found that the somewhat lowered oxygen uptake shown by avitaminous tissue (optic lobes and the lower part of the brain) was partially restored when a vitamin-B₁ concentrate was added. They conclude from this that vitamin B itself acts directly upon the oxidative mechanisms involved, and not through the agency of any secondary substance which it may set free. In the second paper (Gavrilescu, Meiklejohn, Passmore & Peters), however, they find that the oxygen uptake of avitaminous pigeon brain is not notably less than the normal. It is only when lactic acid and glucose are added to it that a subnormal oxygen uptake is readily demonstrable. Addition of a preparation of vitamin B₁ increased the uptake. The conclusion drawn was that vitamin B₁ plays a specific rôle in the intermediate metabolism of carbohydrate, namely in the glucose-lactate enzyme system.

A general difficulty in the past in investigating the pathology of vitamin-B deficiency has been to know how much the observed ill effects were due specifically to lack of vitamin B and how much to

general undernourishment resulting from the loss of appetite. Even the so-called "neuritic," or neuratrophic, changes are not specific but are seen also in starvation (see, e.g., current work by Woppard). This difficulty is not entirely absent even in considering carbohydrate metabolism. For inanition or starvation, like vitamin-B deficiency, also causes carbohydrate intolerance (Goldblatt & Ellis) and hyperglycaemia, with an increased C:N urinary output (Roche). The balance of evidence, however, seems to suggest that the defect in carbohydrate metabolism is caused directly by vitamin-B deficiency, the ill effects in question being probably more pronounced than those seen in general undernutrition.

The question of specificity and differentiation between effects due specifically to vitamin deficiency and those due to underfeeding have been examined by Sure and his collaborators, using the technique of paired-feeding controls. A long series of papers has been published, and the general conclusion reached is that vitamin B has a direct specific effect, unrelated to food intake, upon the following functions—growth, lactation, water metabolism, and glycogen formation [Sure & Smith, (1), (2); Sure, Kik, Smith & Walker; Sure, Kik & Smith]. Lipaemia was at first regarded as another specific effect [Smith & Sure; Sure & Smith, (3)], but this has since been withdrawn [Sure, Kik & Church, (1) and (2)]. Lymphopaenia with polymorphonuclear leucocytosis is also claimed as a specific effect (Sure & Walker). Opinion is, however, by no means unanimous in regarding these effects as specific. Thus deficient glycogen formation cannot be regarded as specific to the vitamin deficiency according to a detailed study by Osuka. Again, conclusions are very conflicting as to whether vitamin B has a direct action on growth. Griffith & Graham found that the appetite failed on diets deficient in vitamin B₁ (or B₂) before the food utilisation was affected. The growth rate, in fact, depended on the caloric intake, considerable variations in vitamin intake having but little effect. Similarly, prolonged experiments on dogs by Cowgill and his collaborators have also led them to the conclusion that the various ill effects noted were due to the anorexia to which the vitamin deficiency gave rise and not directly to the vitamin want itself.

According to Cowgill, the vitamin-B requirement is related to the fuel value of the food consumed. Forced exercise shortened the time required for bringing on the symptoms, a result which was held to support the contention that vitamin-B requirements are pro-

portional to the metabolism (Cowgill, Rosenberg & Rogoff). In a further paper it is agreed that there is a decrease in carbohydrate tolerance (as measured by the blood-sugar curve) in avitaminosis, but it is maintained that this is the result of the inanition, and the theory that vitamin B₁ plays a specific rôle in carbohydrate metabolism is rejected (Burack & Cowgill).

Cowgill and his co-workers have made an examination of the relative vitamin-B needs of different species, as a result of which they deduce the formula

$$\text{Vitamin required per day} = K_s \times \text{wt}^{5/3}$$

where K_s is characteristic for the species and depends on the maximal normal weight of the individual. The formula is utilised for calculating the theoretical human requirement (Cowgill, Deuel, Smith, Klotz & Beard).

Isolation of vitamin B₁.—Interest at the moment centres largely on the crystalline vitamin-B₁ preparation isolated by Windaus and his colleagues, and on the question whether this material is identical (as now appears likely) with that obtained some years previously by Jansen & Donath. The great novelty about Windaus' preparation was that it was shown to contain sulphur, which had not previously been recognised as a constituent of the vitamin. His actual claim to have isolated vitamin B₁ was based on an apparently greater activity than any previously recorded. It has since been shown, however, that Jansen & Donath's preparation likewise contains sulphur, which had been overlooked; and the two are probably identical. There is, however, still some difference of opinion as to the exact elementary composition of the material [van Veen, (5); Tschesche; Odake, (2)].

ANALYSIS OF CRYSTALLINE VITAMIN-B₁ PREPARATIONS

Windaus <i>et al.</i>	$\text{C}_{12}\text{H}_{17}\text{N}_3\text{OS}$
van Veen, (5).....	$\text{C}_{12}\text{H}_{20}\text{N}_4\text{O}_2\text{S}$
Odake, (2)	$\text{C}_{12}\text{H}_{16}\text{N}_4\text{O}_2\text{S}$
Cf. Jansen & Donath's original formula.....	$\text{C}_6\text{H}_{10}\text{N}_2\text{O}$
van Veen, (1).....	$\text{C}_6\text{H}_{10}\text{N}_2\text{O}_2$
Odake, (1)	$\text{C}_6\text{H}_8\text{N}_2\text{O}_2$

The process adopted for the isolation by Windaus and his collaborators (Windaus, Tschesche, Ruhkopf, Laquer & Schultz) followed conventional lines. Yeast extract was the raw material. The vitamin was adsorbed first on fuller's earth, and afterwards foreign substances were removed by successive treatment with HgSO_4 , AgNO_3 ,

and benzylation. The final stages were the preparation of a gold salt, followed by decomposition with H_2S , and crystallisation as picrolonate—which was found to be dimorphous. The isolated picrolonate was converted into hydrochloride. Elementary analysis gave the result entered above. Attention was drawn to the presence of sulphur, and it was concluded that “the crystals have the greatest activity of all previous vitamin-B₁ preparations. We think, therefore, that we have prepared pure vitamin B₁ from yeast.” The minimal daily pigeon dose was given as 1.4–3.3γ per day, compared with the value of 7–9γ determined by the Oxford workers upon Jansen’s crystals (Jansen, Kinnersley, Peters & Reader). The difficulty about accepting these activity values is to know whether the tests were carried out under sufficiently comparable conditions. Jansen himself had originally determined the activity of his material on rice birds, it will be recalled. No doubt a direct comparison of the potency of the two substances will soon be forthcoming.

Van Veen, (1–5), starting with rice bran, has obtained as his final product a preparation which appears to be identical in every respect with that obtained by Windaus from yeast [van Veen, (5)]. It had the same biological activity, and a mixed melting-point determination showed no depression, and both formed HCl salts, chloroaurates, and picrolonates with the same melting-points. Van Veen concludes, furthermore, that both substances are probably identical with Jansen & Donath’s vitamin crystals (obtained from rice). This is confirmed by Tschesche’s direct observation that Jansen & Donath’s crystals, like Windaus’, contain sulphur, and that the mixed melting-point of the two is not depressed. Odake, independently working on rice bran, has obtained a crystalline material, which contains sulphur, and has a minimal pigeon dose of about 0.01 mg. per day. He works out that the amount of the vitamin present in rice bran is 0.0003 per cent.

Simpson confirms that Jansen & Donath’s method gives a highly potent material (active in doses of 0.01 mg.) but points out that impurities are present in it.¹

¹ Since the above was written, Kinnersley, O’Brien & Peters, (1 and 2), have brought forward evidence that specimens of vitamin-B₁ crystals prepared by them are one and three-quarters times as potent as those of Windaus and can be still further fractionated. They conclude, therefore, that Windaus’ preparation cannot be the pure vitamin. The possibility that this difference in activity may be due merely to Windaus’ preparation being partly in an inactivated condition is not discussed.

As this review is being prepared for the press, the very surprising assertion is made by Guha & Chakrovorty, in a preliminary communication to *Nature*, sent by cable, that vitamin B₁ can be synthesised photochemically by the ultra-violet irradiation of adenine sulphate.

Chemical and physical properties of vitamin B₁.—Crystalline vitamin-B₁ hydrochloride as examined by Windaus showed no Pauly reaction. It gave a strong lead test for sulphur. It was precipitated by phosphotungstic acid, AuCl₃, AgNO₃ + Ba(OH)₂, picrolonic, rufianic, and reinicke acids, and H₂PtCl₆ in alcohol; but not precipitated by picric, stypnic, or flavianic acids, HClO₄, AgNO₃, HgSO₄, PbSO₄, or H₂PtCl₆ in water. Its melting-point was 245°; Odake, (1), found 250° for his preparation. The sulphur linkage is not that of cystine. The X-ray crystal-structure method shows that the vitamin hydrochloride has a large flat cell, while the strong negative birefringence points to a ring molecule (Bernal). An ultra-violet absorption band at 250–260 m μ noted by Damianovich and by Guha is confirmed in the paper by Windaus and his colleagues. Bowden & Snow found bands at 260, 240, and 210 m μ and quote a result which might conceivably mean that the one at 260 is correlated with vitamin activity (cf. Heilbron & Morton). Heyroth & Loofbourow point out that the absorption spectrum resembles that of purines and pyrimidines, and that the latter are present as impurities in most preparations.

Occurrence of vitamin B₁ in milk.—The poverty of milk in vitamin B probably comes as a surprise to most people—including those biochemists who may recall that Hopkins' pioneer work on vitamins was based on his finding that a very small amount of milk sufficed as a source of accessory factors when added to a purified basal diet. The explanation of the anomaly is not yet clear. Gunderson & Steenbock show that the vitamin-B value of the milk, always poor, is unaffected by the breed of the cow or its diet, and they suggest, therefore, that it may be under some kind of physiological control. Similar results were obtained a few years ago by the writer. In conformity with the foregoing is the finding of Samuels & Koch that 25 cc. of cow's milk were needed to provide a rat with an adequacy of vitamin B₁, representing 3.5 gm. of the milk solids (from one-sixth to one-fifth was destroyed during the process of commercial evaporation). A further demonstration of the low vitamin-B value of cow's milk is provided by the observation of Catel & Pallaske that goats were unable to survive on a diet of cow's milk, unless it were supplemented

with yeast or lemon juice. In explanation of this last result, it should be mentioned here that Abderhalden has shown that raw goat's milk is a better source of the vitamin than cow's milk. That the ration has little effect on the vitamin-B₁ value of cow's milk has been shown also by Hunt & Krauss, and by Kieferle, who tried the effect of feeding yeast to the cow. Sheep's milk is likewise poor in antineuritic activity (Lawrow).

Occurrence of vitamin B₁ in fruits and vegetables.—Roscoe found that the orange had the highest activity of a number of fruits tested, being one-fifth as good as yeast and equal (on the basis of dry weight) to green-leaf vegetables or liver. The tomato had less than one-tenth the potency of yeast, the banana one-twentieth, and the apple was still lower.

Occurrence of vitamin B₁ in cereals.—Aykroyd has made an interesting study of "parboiled" rice, to account for its vitamin-B₁ activity. (In the process of parboiling, the rice is first soaked in water, then steamed and dried in the sun, and finally de-husked. Beri-beri is not seen in communities subsisting on this "parboiled" rice, in place of ordinary polished rice.) It used to be supposed that the presence of the vitamin in the "parboiled" rice was due to an incomplete removal of the pericarp, but Aykroyd has now shown that the true explanation is that the vitamin gets washed into the endosperm from the pericarp during the course of the process. The "phosphorus index" which generally runs roughly parallel with the vitamin activity of a cereal preparation, and is therefore sometimes taken as a guide, is deceptively low for "parboiled" rice.

VITAMIN B₂

Pellagra in human beings.—Wheeler & Sebrell have published a review dealing with the occurrence of pellagra in the United States. They say that no fewer than 7,146 deaths occurred from the disease in 1930, 98 per cent of these being in the cotton belt. It is now possible to keep it fully under control in institutions. Thus in one of the largest insane asylums the mortality has been reduced in recent years (by dietary treatment, of course) from 6.2 per cent to less than 0.1 per cent. Fundamentally the problem is an economic one, which, it is suggested, can finally be solved only by control of the cotton crop. Probably pellagra was prevalent in the United States as long ago as 1885, but it was not recognised until the 1907-8 outbreak [Wheeler, (1)].

Pellagra is frequently encountered in the Soviet Republic of Georgia (Kandelaki). In Tashkent (Russian Turkestan), seventy-four cases were seen at the Hospital for Tropical Diseases during 1930-31. The mortality rate was 28 per cent (Kassirsky & Burova). In Hyderabad, in the Deccan (India), forty cases have occurred at a leper hospital (Lowe). Two sporadic cases have been reported in the Tirol (Kumer), three cases at Basle (Meyer), and one case (with fatal termination) at Geneva (de Mossier & Starofinski).

Avitaminosis B₂ in experimental animals.—A difficulty in the past has been to obtain regular symptoms of dermatitis in rats suffering from vitamin-B₂ deficiency. Hogan & Richardson claim to have succeeded in doing so by using ultra-violet irradiated yeast as the source of vitamin B₁ (or additional B factors other than the anti-dermatitis). This apparent instability of vitamin B₂ to ultra-violet light needs more extensive study, however, if it is to be correlated with earlier studies on the effects of irradiation upon vitamins B₁ and B₂.

Concentration of vitamin B₂.—Levene has described a process by which concentrates of both vitamins B₁ and B₂ may be obtained from brewers' yeast, the latter being found in the residue after his silica-extraction process. The rat dose was 0.5 mg. per day.

Chemical and physical properties of vitamin B₂.—Halliday, Nunn & Fisher have determined the stability of vitamin B₂ to heat at different pH values. Protein-free milk was used as the source of the vitamin, and the percentage destruction (as determined from results of growth tests and by anti-dermatitis action) was measured after heating for one and four hours at pH 4.3, 7, and 10. It was found that the loss of activity increased very rapidly with increasing pH. In the cold at pH 10, 75 per cent of the activity quickly disappeared, but there was no loss in the cold at pH 7 or 4.3. Samuels & Koch found no appreciable destruction of B₂ during the commercial evaporation of milk.

Vitamin-B₂ assay.—In determinations of vitamin B₂ no less than of B₁, coprophagy has to be guarded against, since it is known that the usual ill effects fail to appear when rats are allowed access to their faeces. Guerrant & Dutcher have made a quantitative study of the influence of coprophagy in this connection.

Distribution of vitamin B₂: plant sources.—Wheeler, (2), has discussed the use of different vegetable materials in their practical application for the prevention of pellagra. Canned spinach was not

a particularly rich source of the "P.P." factor. Canned turnip greens, although only moderately potent, is recommended as a practical and efficacious dietary supplement in pellagrous districts. Canned green beans were relatively poor, and mature onions very poor.

Roscoe has tabulated the vitamin-B₂ values of different fruits and vegetables. She found orange, tomato, and banana to possess rather less than one-tenth the value of yeast, and apple had about one-twentieth the value (on a dry-weight basis). Green leaves, like liver, meat, and egg-yolk, were relatively richer in B₂ than in B₁, while orange juice, like wheat germ and whole cereals, on the contrary, was richer in B₁ than in B₂. Cereal products have been investigated by Hetler, Meyer & Hussemann. Oats, maize, or wheat had to be fed at as high a level as 50 per cent of the food intake to provide enough vitamin B₂ for normal growth in rats (cf. 25 per cent for vitamin B₁). Various oat products were assayed also, and it was found that the kernel was more potent than the endosperm. Rice polish and tikitiki were shown to be relatively poor sources. Wan confirms the moderate activity of soy beans, equal to one-third of that of milk. Peanuts are better as a source of B₁ than of B₂ (Sherwood & Halverson).

Distribution of vitamin B₂: animal sources.—The vitamin-B₂ value of milk has been examined by a number of investigators. Samuels & Koch point out that, while the amount of milk needed to provide a sufficiency of vitamin B₂ is somewhat less than that for B₁, it is yet fairly large, namely from 17 to 20 cc. per rat per day (or from 2.4 to 2.8 gm. of total solids). Commercial evaporated milk was equal to fresh milk. Human milk has been tested by Donelson & Macy with similar results. Five cc. per day of human milk gave some degree of growth, but as much as 20 cc. was not enough for maximal growth. These authors suggest that in some cases the low vitamin-B₂ value may be the "limiting factor" in breast milk.

ADDITIONAL "B" AND UNCLASSIFIED FACTORS

"Physin" and the promotion of lactation.—Mapson, working at the Biochemical and at the Nutritional laboratories, Cambridge, has made a study of a substance present in liver which he has named "physin," which has a remarkable stimulating action on growth rate, on lactation, and on the reproductive performance of rats. Perhaps the most striking feature of physin, however, is that its effect is transmitted from parents who have received liver to their offspring

who have never had any. This action upon the offspring, i.e., in giving rise to a supernormal growth rate commencing suddenly at adolescence, is in fact more marked when the mother has been fed on the liver (i.e., before or during gestation and lactation) than when the offspring receive it direct themselves. The basal diet used in these experiments was complete in all the hitherto recognised dietary essentials, and physin itself appears to be clearly differentiated from them all. Little is yet known, however, about its chemical characteristics.

A substance which stimulates lactation in rats and improves the growth rate of the young has also been examined by Wilkinson & Nelson. The basal diet contained soy bean as the sole source of vitamins B₁ and B₂, and the new factor was found to be provided by the addition of liver, kidney, pancreas, brain, or heart, but not by lung, spleen, or thymus. This factor, as examined in liver, is thermolabile and ether-insoluble, and was shown also to stimulate the growth of the suckling young when fed to the lactating mother.

Leg-paralysis factor for chicks.—A peculiar type of paralysis affecting the legs and feet was noted by Norris, Heuser & Wilgus in chicks which had been fed on a restricted diet. The paralysis could be prevented by the inclusion in the diet of the following substances, which are therefore held to contain the new anti-paralytic factor: milk powder or vitamin concentrate from milk, yeast, autoclaved yeast, and alfalfa meal. The following were inactive: meat scraps, fish meal, whole meal (Norris, Heuser & Wilgus; Norris, Heuser, Wilgus & Ringrose). The factor is described as heat-stable. Bethke, Record & Kennard have confirmed Norris' findings and are satisfied that the paralysis of the feet and legs is not due to deficiency of any known vitamin or major dietary constituent. Hart, Keltzien, Scott & Halpin observed symptoms in chicks apparently identical with those described by Norris, but were unable to prevent them by feeding skim milk or skim-milk powder and therefore do not agree with Norris' conclusion that there is a new vitamin in milk.

"B" growth factors for rats and chickens.—Halliday, Nunn & Fisher, (2), have noted that rats receiving vitamins B₂ and B₁ in the forms, respectively, of autoclaved yeast and skim-milk powder (or protein-free milk, or activated Lloyd's reagent prepared from it) grew better when whole wheat was added to the diet. This is held to support Reader's views on the existence of a factor B₄, which is presumed to be present in the whole wheat. Daggs & Eaton found that

rats fed on a vitamin-B-free basal diet supplemented with large amounts of canned tomato juice developed dermatitis. With smaller quantities (from 6 to 7 cc.) of tomato juice, growth failure occurred instead. This was attributed² similarly to shortage of vitamin B₄.

The conclusion that vitamin B₂ is of a complex nature has been reached by a number of workers who have observed a lack of parallelism between anti-pellagrous and growth-promoting activity in tests on rats [Halliday, Nunn & Fisher, (1); Sure, Kik & Smith], or on chicks (Ringrose, Norris & Heuser).

Miscellaneous.—Other new factors have been claimed by Pappenheimer & Goetsch (preventive of cerebral disorder in chicks), Roscoe (stimulating vitamin-B synthesis in coprophagy), Parsons (preventive of egg-white injury), Szmanska (insoluble fraction of egg-yolk), and Rosenow (alleged "growth factor of hay"; cf. Scheunert).

In last year's review the evidence for some of the newer factors was subjected to a rather critical discussion. We shall only observe now that much of the more recent work seems also to have been published at a very preliminary stage; that the symptoms described are often quite vague or unspecific (e.g., diminished growth rates); that the basal diets in several cases were themselves apparently deficient in certain known factors; and that, in general, little effort is made to give a satisfactory differentiation from the factors of other workers or indeed sometimes even from the better-known vitamins themselves.

VITAMIN C

Scurvy in human beings.—Scurvy is fairly common among the natives, children and adults alike, in South Africa, and Ross refers to a series of 208 cases, 9 of whom succumbed and 199 recovered. Gedda has measured the "vitamin-C standard" of students at Upsala by means of the capillary-resistance test of Göthlin and finds it to be lower in the spring than in the autumn, corresponding with the diminished vitamin-C intake during the winter.

Experimental scurvy.—The writer with J. R. M. Innes has shown that dogs, unlike guinea pigs or human beings or monkeys, but like

² Note added to proof: Barnes, O'Brien & Reader have just reported a crystalline preparation, apparently of constant chemical composition, possessing vitamin B₄ activity.

rats, are able to synthesise their own vitamin C when none is present in the diet, and so are not susceptible to scurvy. Brouwer & de Ruyter de Wildt have determined the vitamin-C requirement of goats and find it to be very small. Monkeys receiving a diet devoid of vitamins A and C developed scurvy in from 70 to 80 days, which responded promptly to treatment with orange juice (Turner & Loew). Harris & Ray have shown that the suprarenal gland of a normal guinea pig is active to a remarkable degree as a source of vitamin C, but that with the onset of scurvy the activity disappears.

Vitamin C and hexuronic acid.—An outstanding advance of the year has been the demonstration of anti-scorbutic activity for Szent-Györgyi's hexuronic acid. This substance was first isolated as a reducing principle present in various plant and vegetable tissues, orange and lemon juice, cabbage, suprarenal cortex, etc. [Szent-Györgyi, (1)]. Its similarity in distribution and properties to vitamin C was commented on by Szent-Györgyi from the first, but it was contended by Zilva that anti-scorbutic activity bore no constant relationship to reducing capacity, and the matter was dropped for some years. In the meantime in a series of papers Tillmans and his collaborators from Frankfurt pointed out that there did in fact appear to be some definite correlation between the reduction potential of foods and their vitamin-C content. Early in 1932 Szent-Györgyi, in collaboration with Svirbely (who, it may be recalled, had lately been associated with King in work on vitamin-C concentration at the University of Pittsburgh), announced that a daily allowance of 1 mg. of hexuronic acid would indeed protect guinea pigs from scurvy over a period of 50 or 90 days [Szent-Györgyi, (2); Svirbely & Szent-Györgyi, (1), (2), (3)]. Szent-Györgyi concluded from this evidence that "vitamin C is a single substance identical with hexuronic acid." Independently and simultaneously, King and his collaborators, who had been continuing their work on the concentration of vitamin C (see Svirbely and King; Smith and King), announced that their final crystalline vitamin preparation, which reached a constant anti-scorbutic activity (minimal daily dose = 0.5 mg.), had all the chemical and physical properties of Szent-Györgyi's hexuronic acid (King & Waugh; Waugh & King). Zilva, (1), still combats Szent-Györgyi's conclusions, maintaining that he has himself handled fractions which are even more active than hexuronic acid and still grossly contaminated. He admits, however, that specimens of hexuronic acid may contain some vitamin C [Zilva, (2)]. This question

has been investigated by the writer and his colleagues, and some interesting developments have resulted. In particular the possibility had to be examined whether the activity of hexuronic acid might possibly be due not to the main constituent but to some contaminating impurity. In the first place, the anti-scorbutic activity of hexuronic acid was duly confirmed (Harris, Mills & Innes). This was done not by the usual protective technique but by the more striking method of curative tests and also by the microscopic tooth-structure method. The actual degree of activity of the hexuronic acid was also determined. It was then argued that if hexuronic acid is identical with vitamin C the raw suprarenal cortex, although it had not previously been recognised as possessing anti-scorbutic activity, should in fact be intensely active, since it is the best of the known sources of hexuronic acid. This prediction was borne out by the experimental results (Harris & Ray). In fact, the degree of activity of the suprarenal cortex was precisely that calculated from its estimated hexuronic-acid content. The same was true of many other natural sources examined. It was pointed out, furthermore, that hexuronic acid obtained from different sources possessed the same anti-scorbutic activity. This evidence all strongly supports Szent-Györgyi's conclusions and shows that vitamin C is probably identical with hexuronic acid, or if not it is at any rate a substance which is remarkably closely associated with it in its natural distribution and which follows it persistently in the various isolation processes. There can be no doubt then that this knowledge in itself represents a great advance in our understanding of the chemical nature of vitamin C.

Vitamin C and reducing capacity.—One objection raised by Zilva to the hexuronic-acid hypothesis is that according to his observations vitamin-C activity does not run parallel with reducing activity. As pointed out by Svirbely & Szent-Györgyi, (3), one reason for this may be the occurrence in orange-juice products of phenolic substances having independent reducing capacity. But the more satisfying explanation is found in the work of Tillmans & Hirsch and their collaborators, namely, that while anti-scorbutic activity is in fact characterised by reducing action both are subject to a reversible as well as to the better understood irreversible oxidation process. The independent series of papers by Tillmans *et al.* to which we are now alluding is in admirable conformity with Szent-Györgyi's conclusion, and must now be briefly reviewed.

In a preliminary communication (1930) Tillmans points out that

there is a reducing substance present in natural foodstuffs, which can be estimated by titration against the indicator, 2,6-dichlorophenolin-dophenol, and which appears to run parallel with their recorded vitamin-C contents. Moreover, the reducing substance in question seemed to have a sensitivity towards heat and alkalies agreeing with that of the vitamin. In the first paper of the new series (Tillmans, Hirsch & Hirsch) it is shown that this reducing capacity in lemon juice may be used as a criterion for the separation of anti-scorbutic fractions. The activities of various fractions precipitated by lead acetate similarly ran strictly parallel with their reducing capacities (Tillmans, Hirsch & Siebert). The process is next described in detail whereby various vegetable sources are extracted and titrated with 2,6-dichlorophenolin-dophenol. The titration values so obtained run exactly parallel with vitamin-C content, except only in the presence of copper, which was found to interfere. The reducing substance present in leafy vegetables seemed to be identical with that previously examined in orange juice, and the authors conclude that the indicator is "specific for the reducing substance, which we regard as a carrier of vitamin-C activity." Titration with iodine, on the other hand, was found to be less specific [Tillmans, Hirsch & Jackisch, (1)]. The paper by Tillmans, Hirsch & Dick distinguishes clearly between the reversible and the irreversible stages in the oxidation of the reducing substance. The first stage in the oxidation brought about by I_2 or H_2O_2 or by the indicator is reversible and can be restored by H_2S , which likewise brings back the anti-scorbutic activity. Oxidation by atmospheric oxygen, on the other hand, is irreversible. In the first or reversible stage, if the reversibly oxidised substance is allowed to stand, its reversibility is gradually lost. Observations on cucumber extracts [Tillmans, Hirsch & Jackisch, (2)] confirmed these conclusions: the reducing substance in cucumber was shown to be more readily oxidised on standing than was the case with orange juice, but after treating the extract with H_2S (to reverse the oxidation) it could be accurately assayed, and there was the usual correlation between anti-scorbutic activity and reducing titre.

Zilva, (3), has recently criticised Tillmans' work on the grounds that his own observations collected over many years fail to show a parallelism between the anti-scorbutic activity of various lemon-juice fractions and their reducing capacity towards *l*-dimethylaminophenyl-indophenol. The important issue of a reversible stage in the oxidation, as postulated by Tillmans, he proposes to discuss later.

Methods used in concentration of vitamin C.—Earlier papers by Svirbely & King and Smith & King describe minor developments in King's procedure; and in the final paper by Waugh & King the steps, starting with lemon juice as the raw material, were essentially as follows: decitration, solution in *n*-propyl alcohol, precipitation of impurities by petrol ether, extraction with ethyl acetate (or an alternative precipitation process with lead acetate), and, finally, precipitation at low temperature by means of petrol ether, followed by re-crystallisation by addition of petrol ether to solutions held in either butyl alcohol, or acetone, or ethyl acetate or methyl alcohol.

It was claimed in the earlier stages that the degree of concentration effected was from 0.03 to 0.5 mg. (Svirbely & King) or 0.09 mg. (Smith & King) of total solids per 1 cc. of original lemon juice, but there would appear to be some discrepancy here, for the later result shows that the final and most concentrated product (hexuronic acid) has a minimal dose of not less than 0.5 mg.

Tillmans' process (Tillmans, Hirsch & Hirsch) involved, as its essential features, decitration, extraction with acetone, and precipitation with barium and lead.

*Chemical properties of vitamin C or hexuronic acid.*³—The chemical and physical properties of the most concentrated preparations of

³ *The alleged identification of vitamin C with a narcotine derivative.*—Rygh has claimed that vitamin C is identical with methyl*nornarcotine*, and as his conclusions appear to have gained so wide a credence [e.g., editorial, *Nature*; Armstrong; Hopkins; Hess; Bezssonoff] some discussion of his work is needed here. His conclusions seem to the present writer to be based on a series of fallacies; and, moreover, they entirely lack independent confirmation. Rygh [see Rygh, Rygh & Laland; Rygh & Rygh, (1); Laland; Rygh] stated that, starting with the idea that vitamin C is a base, he had extracted orange juice repeatedly with ether and found the extract to be active. With unripe oranges the extract was found to be less active, and a substance present in it, he claimed, was identified with narcotine. It was assumed that, during ripening, by the action of ultra-violet light vitamin C was generated from its precursor, narcotine. A specimen of narcotine was therefore irradiated and tested for anti-scorbutic activity, as also were various artificially prepared narcotine derivatives, including methyl*nornarcotine*. Irradiated narcotine and methyl*nornarcotine* were thought to be active, since a group of animals receiving a particular level of each were judged, when autopsied, to be free from scurvy. They did not, however, survive any longer than the negative controls receiving no vitamin C. This was attributed to the dual nature of vitamin C, and to unknown deficiencies in the basal diet which were rectified by the addition of heated fruit juice—whereupon the animals survived. However, when the alleged active materials were given at levels either slightly above or below the supposed optimal levels

vitamin C, as obtained by King and by Tillmans and their collaborators, have properties identical with those described for hexuronic acid so far as is yet known—e.g., solubility in water, methyl alcohol, ethyl alcohol, acetone; insolubility in fat solvents; precipitation by lead; sensitivity to oxygen, especially in alkaline solution; absence of nitrogen; and presence of the carboxyl group. According to Waugh & King there are two forms of the pure vitamin C, free acid and lactone (but cf. Cox, Hirst & Reynolds). The m.p. was 183°–185°,

the animals were no longer protected. It was concluded from this that the vitamin has an "optimal point of action."

The following criticisms seem pertinent: (1) The presence of some vitamin C in the wet ether after repeated extraction would by no means prove the basic nature of vitamin C—in fact, all the evidence points very strongly to its being acidic; and vitamin C is, in fact, virtually insoluble in moderate amounts of dry ether. (2) The assumption that the synthesis of vitamin C in plant life depends on the action of ultra-violet light is erroneous, as was shown some years ago by Eggleton and the present writer. (3) The identification of narcotine in the unripe orange extract seems uncertain. (4) But perhaps the most unconvincing feature was the failure of the animals receiving the alleged active preparations to survive any longer than the negative controls: their apparent freedom from scorbutic lesions may perhaps be attributable merely to some irregularity in the date of the first appearance of the symptoms—such as, in our experience, is not unusual. (5) The suggestion that an anti-scorbutic material has no longer any protective action when given in amounts slightly above the optimum runs counter to all previous experience. (6) The improved results observed by Rygh when heated fruit juice was given can be attributed to the presence in it of a certain amount of vitamin which had resisted destruction. (7) Very astonishing is the assertion that vitamin C, so sensitive to heat, is prepared by boiling narcotine for several days with concentrated acid. (8) Methyl*nornarcotine* was also supposed to be produced from narcotine *in vitro* under the influence of the mercury vapour lamp; but such a *demethylating* action for the ultra-violet rays seems contrary to all previous ideas of photochemical action.

Attempts have been made in the writer's laboratory to repeat Rygh's work, but no anti-scorbutic activity could be detected for either irradiated narcotine or for methyl*nornarcotine* (Dann, Forsyth, Harris, Mills & Innes). Negative results with the latter substance have been recorded also by Brüggemann and by Grant, Smith & Zilva (see also Smith & Zilva), who were unable to detect any narcotine in oranges; and Dalmer & Moll could not confirm Rygh's results in any respect. Tillmans & Hirsch point out that methyl*nornarcotine* has not the reducing action which they hold to be characteristic of vitamin C. (See also discussion by Ott & Packendorff as to the question of the presence of narcotine in lemon juice, and the reducing activity to Tillmans' indicator of the water-soluble ether extract.)

Quite recently a further paper by Rygh & Rygh, (2), attempts to account for the observed activity of hexuronic acid by assuming that it contains methyl-

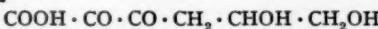
and the optical rotation $[\alpha]_D^{20} = 25^\circ \pm 1^\circ$, and the C and H combustion and acidimetric and iodine titrations agreed with the formula $C_6H_8O_6$ (Waugh & King).

Hexuronic acid has a single broad band at 263 m μ (Bowden & Snow; Herbert & Hirst), which resembles that given by many ketonic substances, but differentiates it from an aldose or ketose sugar of the pyranose type, and was thought to render unlikely the formula earlier proposed (Hirst & Reynolds) with a keto-furanose structure and carboxyl group in position 6. X-ray examination and measurement of birefringence suggest that the hexuronic-acid molecule has a ring structure with fewer groups projecting than is the case with a normal carbohydrate, and that it contains double-bonds possibly in carboxyl groups (Cox).⁴

nornarcotine as an impurity, which it holds tenaciously, and to which it owes its vitamin-C activity. Methyl*nornarcotine* and hexuronic acid are supposed together to be the two constituents of a vitamin-C complex. In evidence of this, curves are reproduced showing that guinea pigs receiving simultaneously methyl*nornarcotine* plus glycuronic acid (assumed [*sic*] to have similar functions to hexuronic acid) remained free from scurvy and grew normally. Tests in the Nutritional Laboratory, Cambridge, have entirely failed to confirm this statement (Dann). This latest development in Rygh's theory, therefore, while ingenious, and in parts difficult of disproof, does not meet the earlier criticisms (see Harris, Mills & Innes), and like his earlier work it cannot be reproduced experimentally.

As yet further evidence for his theory, Rygh has advanced the considerable claim that he has succeeded in curing seven cases of scurvy in human beings by treatment with methyl*nornarcotine*. (In the human, unlike the guinea pig, he believes that the addition of hexuronic acid is unnecessary.) We think it sufficient here to point out that in the one case out of the seven which has so far been fully reported (Römkke & Rygh) the patient received a diet which included pasteurised milk, meat cakes, and boiled fish, the first constituent of which certainly, and probably the second also, would contain quite appreciable amounts of vitamin C.

* As this review goes to press, Cox, Hirst & Reynolds have suggested the structural formula



which can also react in the enolic form



This formula satisfies the requirements of the X-ray and crystallographic data, and accounts for the observed reactions. They include the following: oxidation by I₂, NaIO₄, and acid KMnO₄; formation of diphenylhydrazone and di-*p*-bromo-phenylhydrazone; condensation with *O*-toluylene diamine; coloration of the sodium or calcium salts with FeCl₃ or sodium nitroprusside; formation of

The determination of vitamin C.—The writer has introduced a curative method which has a number of advantages over the usual preventive test. Thus a determination can be concluded within little more than a week instead of the usual 90-day period, and is therefore less extravagant of material, as well as being more convenient. The accuracy is as good or greater than that of the usual method (Harris, Mills & Innes; Harris & Ray).

Vitamin C: distribution, origin, and effect of canning, storage, and other treatments.—No less than from forty to fifty papers dealing with these questions have appeared during the year. Many of these papers really deserve a far more detailed notice, but we can do no more than draw attention to some of the more outstanding results. As to the origin of the vitamin, the conclusion reached some years ago by Eggleton & Harris that vitamin C can be synthesised during germination in the absence of light is confirmed (Matsuoka). (It had previously been supposed that the diminished incidence of infantile scurvy in summer denoted the synthesis of the vitamin under the action of light.) Again there is little evidence of any considerable seasonal variation in the anti-scorbutic value of cow's milk: it has yet to be shown whether the cow is able to make its own vitamin C independently of the diet (de Ruyter de Wildt & Brouwer; Gabathuler). An interesting genetic relation has been shown to exist between the anti-scorbutic activity of different varieties of apples, depending on their chromosome number (Crane & Zilva); and similar work is being done comparing different varieties of spinach (Kifer & Munsell), mangoes (Perry & Zilva), and other materials. Oranges from different parts of the world have been found to show an unsuspected variation, the Indian and Chinese varieties being far less potent than the Western types (Wats & White; Oliveira). Elaborate surveys have been carried out of the vitamin-C resources of different parts of the world, including Germany [v. Hahn, (2),

triacetate ester and trimethyl ether; loss of CO₂ on heating; higher rotation of sodium salt than free acid (keto-enol transformation); broad absorption band at 265 mμ.

The following physical properties are given: On rapid heating, m.p. = 192°, but loses CO₂ at 175°. $[\alpha]_{5780}$ for free acid = +24°; for sodium salt = +105°; for sodium salt in N/10 alkali = +135° (increased rotation of free acid on standing due to alkali dissolving from glass vessel). Alkaline solutions are stable in absence of oxygen. The biological activity is thought to be due to the double oxidation-reduction properties of the acid. An alternative furane ring structure has since been advanced by Micheal & Kraft.

(3)], China (Oliveiro), and India (Wats & White). It has been discovered that the following materials are pre-eminent in vitamin-C activity: suprarenal cortex (notable as an animal source [Harris & Ray]), certain varieties of mango (Perry & Zilva), and the pumelo (Wats & White); a good source is sheep's liver (Mills); poor sources are the olive, peach (Newton; cf. earlier results), Indian pear (Wats & White), and pomegranate (Wats & White); while a shell fish is included among foods devoid of the vitamin (Miller). Somewhat conflicting results have been reported for the vitamin-C activity of bananas [v. Hahn, (1); Wats & White; Stanley], and more particularly of cranberries (Boguliubora). The official method of processing oranges employed by the United States Plant Quarantine and Control Administration, for destroying the larvae of the American fruit fly (by heating for eight hours at 110° F.), does not affect the vitamin-C activity, but spraying the trees with lead arsenate does (Nelson & Mottern). Preserved apples and most conserves, with the exception, it is stated, of spinach (Remy), rapidly lose their vitamin-C activity. The effect of many canning, preserving, and cooking processes has been investigated, and undoubtedly varies immensely from one food to another and from process to process. Liver loses its anti-scorbutic activity on freezing, apparently owing to the action of enzymes or other substances set free by the disintegration of the cells (Mills). Johnson & Zilva think that when orange juice is boiled a substance of a catechol-like nature is formed from the sugar which tends to increase the rate of destruction of the vitamin. Remy has emphasised the importance of copper as a factor tending to destroy the vitamin-C activity of preserved foods; other factors, of course, include oxygen and a high pH value.

PART II. FAT-SOLUBLE VITAMINS

VITAMIN A

Vitamin-A deficiency in human beings.—Vitamin-A deficiency is so common throughout India that it is the chief cause of early blindness (see Kirwan; Naseeruddin). As Brahmachari points out, the only two fats used (in Bengal) are mustard oil and ghee, the latter containing very little vitamin A and the former none at all. In Yucatan, where poverty is widespread, one child out of every five of the poorer classes suffers from xerophthalmia (Carillo). In Java and Sumatra keratomalacia is prevalent among the native children and

thirty-five cases were admitted to the Batak hospital in the last two and one-half years (de Haas). Other papers have described cases of vitamin-A deficiency in Peiping (Pillat & Chang), in the state of Louisiana (Buffington), and elsewhere.

Vitamin-A deficiency in chicks and monkeys.—Vitamin-A deficiency in chickens has been investigated by various workers. This species does not readily show any ophthalmic lesions, but characteristic features are (*a*) inco-ordination and (*b*) an accumulation of uric acid in the blood. The latter, as Elvehjem & Neu have shown, is due to kidney damage, and not to any disturbance in metabolism.

Monkeys deprived of vitamin A did not exhibit keratinisation or infections of the eyes and the upper respiratory tract, characteristic of the avitaminosis in most species so far examined, but died with gastro-intestinal symptoms, including the presence of a mucus-like exudate and severe diarrhoea. At post-mortem there is marked enteritis and dilatation of stomach (Turner & Loew).

Mode of action of vitamin A in relation to infection.—A few years ago Mellanby & Green tested the effect of administering vitamin A in cases of puerperal septicaemia. It appeared to have a beneficial effect, and this led to the acceptance in many quarters of the somewhat vague theory that vitamin A was "the anti-infective vitamin." That is, the term was used in a very wide sense to imply that the vitamin is of value, almost indiscriminately, in combating infectious diseases and septicaemias of all kinds. Actually, a series of investigations by the writer in collaboration with J. R. M. Innes and with A. S. Griffith, a summary of which has been recently published, indicates that vitamin A might be more justly described as the "anti-keratinising factor" and that it is "anti-infective" merely in the sense that it prevents the typical keratinisation of membranes which occurs in the absence of the vitamin and leads to secondary infective troubles. In other words, vitamin A does not influence general immunity. It follows from this that there is no basis for the belief that vitamin-A therapy is likely to be effective clinically in acute general infections, caused by specific highly pathogenic organisms. Nor is it likely to be of value in such clinical toxæmias or infectious diseases as are unassociated with the peculiar structural breakdown of epithelial tissues, and the attendant localised infection, which is characteristic of vitamin-A deficiency. The keratinisation of epithelial tissues had been fully described earlier by Mori, Wilson & DuBois, and by Wolbach & Howe. But various workers (Yudkin & Lambert;

Wason, Goldblatt & Benischek; Tyson & Smith; and others) had supposed the infection to precede, or to be independent of, the epithelial changes. In disproof of this supposition, and to show that the infection was purely secondary to the local membrane metaplasia or keratinisation, Harris & Innes followed the slow development of the lesions of a- or hypo-vitaminosis-A in rats. The infections were shown to be of a special type, i.e., limited in origin to epithelial tissues and not seen in the absence of neighbouring keratinisation. "The xerosed membrane being deprived of its normal secretion, micro-organisms are not retarded from settling on its surface; apart from the more physical, or flushing, action of the secretion, its natural antiseptic properties, ascribed to lysozyme, must also be borne in mind. Secondly, the accumulation of a profuse débris of desquamated cells along the surface of a membrane will tend to set up irritation and cause severe obstruction, particularly in narrowly enclosed areas. Good evidence of ducts becoming in fact choked in this way and so rendered liable to infection is found in the experimental pictures of the ducts of the tongue, of the sublingual glands, and of the bronchi; these are commonly seen to be filled with desquamated epithelial cells, distended and infected. The pelvis of the kidney among other sites showed a similar condition." When vitamin A was provided, it was shown that the epithelium became normal again and the local infections disappeared. If the avitaminosis were allowed to develop, the local infections spread, the destruction of the integrity of the epithelium being presumably responsible for absorption of fortuitous infecting micro-organisms, normally non-pathogenic, so that septicaemia resulted. Harris and Griffith carried out experiments to test the suggestion that in vitamin-A deficiency rats lose their normally high resistance to tuberculous infection. Such had been claimed to be the case by Hagedorn, by Grant *et al.*, by Smith & Hendrick, and by others, and this, if confirmed, would support the theory that vitamin A has in fact a real action in increasing immunity to pathogenic organisms, in addition to its function in maintaining the health of the mucous membrane. Negative results were obtained, however, and there was no evidence of any lowered resistance to the spread of tuberculous disease. This is further evidence for supposing vitamin A to be "anti-infective" in the more limited sense only.

Another approach to this problem is to discover whether, in practice, vitamin-A deficiency is specially prevalent among persons

suffering from infectious troubles. This question has been tested by Thomas Moore, (3), at the Cambridge Nutritional Laboratory, who has examined the vitamin-A reserves of human subjects after death. This was done by means of the $SbCl_3$ reaction applied to the liver (in which organ, of course, the body's stores are concentrated), and results were compared for the most varied types of cases, i.e., after death from accidental causes (normal controls), or infectious or non-infectious diseases, or surgical emergencies, etc. Since his results show that in various types of commonly occurring infections, terminated by death, there were often abundant vitamin-A reserves, we have concluded that any advantage to be derived in such cases from the administration of vitamin A—i.e., with the aim of rectifying a non-existing deficiency—must be highly doubtful. On the other hand, it cannot be denied that recent observations indicate that shortage of vitamin A is not altogether rare among certain sections of the community, and of course the importance of providing an adequacy is not to be gainsaid. Perhaps the position may be best summed up by saying that while a low vitamin-A intake, such as would tend to make the subject liable to a special type of infectious trouble, is by no means unknown in human experience, and must be guarded against, on the other hand it is fallacious to regard vitamin A as a "positive anti-infective agent, indiscriminate in action." Moore, (3), found normal ranges of vitamin-A reserves in the following groups of diseases: internal accidents, diseases of the alimentary tract and peritoneum, heart infections, urogenital infections, infections of head and spine (including otitis media), tuberculosis (all forms), neoplastic diseases, and syphilis. In diabetes, the patients having received special dietary treatment, reserves were far above normal. The reserves tended to be subnormal for the following: organic heart diseases, non-tuberculous respiratory diseases (particularly bronchitis), organic kidney diseases, and certain septic diseases. As Moore points out, the low vitamin-A values may sometimes have been a result of the disease rather than its cause, and, contrariwise, high values in certain diseases (e.g., tuberculosis) may have been the result of the intensive dietary treatment and throw no light on the aetiological factors. As has in fact been found by Green of the Sheffield school, and also by Owen & Hennessey, abundant vitamin A given by mouth may fail to make its appearance in the liver in certain diseased conditions. Work similar to Moore's has been reported by Wolff of Utrecht. He finds that 16 per cent of the population of

the Netherlands may be regarded as having subnormal vitamin-A reserves. Low values were found under the following headings: in the newborn and in pregnancy; in embolisms, whooping cough, syphilis, chronic nephritis, and enteric fever. In sepsis, the divergence from the average was "not so distinct." These allocations differ somewhat, it will be noted, from Moore's, and, as Wolff remarks, "in view of the small number of cases . . . the element of chance cannot be completely ruled out." Moore's results and Wolff's are at one in showing how widely the reserves vary from one individual to the next. The *mean* values came out about the same for the two countries.

The views of Lassen must be referred to at this point. Adopting the theory of Orskov as to the anti-infective functions of the reticulo-endothelial system, he infected vitamin-A-deficient rats with paratyphoid bacilli and subsequently made cultures from the different organs with the object of determining their distribution throughout the body. This distribution was such as to lead him to conclude that vitamin-A deficiency leads to an impairment in the activity of the reticulo-endothelial system. However, in keeping with the present writer's views as to vitamin A being anti-infective rather by virtue of its "anti-keratinising" properties, we may note that a much greater difference was apparent between the infectivity of the vitamin-deficient rats and the controls when the micro-organisms were administered by mouth than when they were injected.

Clinical investigations by Barenberg & Lewis failed to show any increased resistance to respiratory infections in infants who were given maximum allowances of vitamin A. In the opposite direction Ellison claims that cases of measles treated with vitamin A (plus D) had a death-rate of only 11 per 300 cases, compared with 26 per 300 in untreated control cases. Further statistical evidence will be needed before this claim can be regarded as fully substantiated, and it may be objected that the "normal" death-rate in the untreated controls appears to be unduly high. Ellison points out, however, that after severe measles there occur metaplastic changes in the respiratory tract resembling those seen in vitamin-A deficiency, which might be regarded as a rationale for the treatment.

Transfer of vitamin A from mother to child.—Work published during the past year goes to show that the vitamin-A reserve of newborn young is surprisingly low and that while it increased during the suckling period there is still a very definite limit to the amount

of vitamin A which can be passed on to the offspring through the milk, no matter how much may be fed to the lactating female. A paper by Dann, (2), deals fully with this topic, rats being used as experimental animals. Somewhat similarly, Busson & Simmonet found that there was no appreciable amount of vitamin A in puppies at birth but that after two months' suckling it might approach one-half the amount in the mother dog. In keeping with Dann's result, may be cited the results of analyses of butters from different sources, carried out by Crawford, Perry & Zilva, which show little variation in vitamin-A contents notwithstanding varied dietary treatment of the cows. Again, the results of Moore, (2), as of Fraps & Treichler, show that, while butter from cows fed on deficient diets was certainly poorer in the vitamin than that from those fed on richer diets, nevertheless only a small fraction of the total vitamin A given to the cow appears in the milk. It would appear therefore that, provided the cow's diet is not really *deficient* in the vitamin, further additions to the diet have relatively little effect on the milk. In the case of human newborn infants, the lowness of the vitamin-A reserves has been noted by Wolff.

Carotene and vitamin A.—Attempts have been continued to convert carotene to vitamin A *in vitro*. Bowden & Snow supposed that this might be accomplished by irradiating carotene with monochromatic light at 265 m μ , the pigment having a weak absorption band in that region. The appearance of a band at 328 m μ was thought to indicate the formation of vitamin A. Woolf & Moore have enumerated the criteria which would have to be satisfied before such a claim could be conceded. As pointed out by Heilbron & Morton, the irradiation of a hydrocarbon like carotene in an inert solvent could not yield an alcohol (vitamin A); and, moreover, the final product of Bowden & Snow was still coloured.

When carotene is fed to a cow it is only partly converted to vitamin A, and is found partly as carotene itself in the liver and body fat [Moore, (2); cf. also Mattikow]. Simmonet & Busson have confirmed the transformation of carotene to vitamin A *in vivo*, in the case of the guinea pig and the dog. According to H. von Euler and Virgin the serum of higher animals contains both carotene and vitamin A.

A large number of papers discussing the natural occurrence of carotene has been published. Euler & Euler show that predatory sea birds and fish can receive but little carotene in their diet and

that if it be given experimentally in the diet, e.g., in the case of *Gobius niger*, little vitamin A appears in the liver.

Much work is now in progress to compare the biological activities and the difference in properties and constitution of the various modifications and derivatives of carotene [e.g., Kuhn & Brockmann, (1), (2), and (3); Kuhn & Lederer, (1), (2); Karrer, Morf, Krauss & Zubrys]. For further discussion refer to p. 397 *infra*.

Isolation of vitamin A.—Progress has been reported by three sets of workers: (a) Moore of the Nutritional Laboratory, Cambridge; (b) Karrer and Euler and Euler and associates in Sweden; and (c) Heilbron and Drummond and their colleagues at Liverpool and University College, London. The products obtained are all of very similar activity, and it seems probable that they now represent the vitamin in a state approaching purity.

The procedure of Moore, (1), was to feed sources of carotene in large quantities to rats and to pigs, thus obtaining liver oils of exceptionally high activity, from which concentrates were prepared along classical lines. As he points out, the final product always approached the same maximum potency, no matter how varied the original activity of the crude liver oil. This suggested either that it was approximately pure vitamin A itself, or else that the vitamin was associated in remarkably constant proportion with some other substance of similar solubility properties.

The most important papers from the Karrer school are two by Karrer, Morf & Schöpp, (1), (2). Earlier communications showed that the original non-saponifiable residue from *Hippoglossus* (halibut) had 6,000 cod-liver oil units ($SbCl_3$ test), and the product obtained by a process of low-temperature fractionation followed by fractional adsorption on Al_2O_3 reached the figure 10,000; the latter was said to have thirteen times (130 times was claimed at first) the biological activity of carotene and from eight to nine times that of "biosterol" [H. von Euler & Karrer, (1)]. A product obtained by treating the *Hippoglossus*-liver oil with anhydrous Na_2SO_4 and ether extraction had only 192 cod-liver oil units, or, after precipitation of the cholesterol, 1,200 units (Karrer, Klussmann & H. von Euler). The very important paper by Karrer, Morf & Schöpp, (1), describes how the unsaponifiable residue from the *Hippoglossus*-liver oil can be freed from sterols by cooling to from -50° to -60° in methyl alcohol, after which follows a process of fractional adsorption on fibrous Al_2O_3 , similar to that used in standard chromatographic

methods. By this means a viscous yellow oil was obtained, with the properties of a carotene derivative, a molecular weight of 320, a minimum rat dose of 0.005 mg., and 10,500 "SbCl₃ units." A similar process was then applied on a larger scale to mackerel oil, viz., sterols separated by low-temperature treatment; solution in petrol ether, adsorption on fuller's earth; elution; esterification as acetate; and saponification of the ester [Karrer, Morf & Schöpp, (2)]. H. von Euler & Karrer, (2), found that, while the concentrate from *Hippoglossus* might show SbCl₃ (Carr-Price) values varying from 50,000 to 100,000, its biological activity remained constant at 0.5γ.

Heilbron, Heslop, Morton, Webster, Rea & Drummond open their report on the note that the papers from their laboratories during the past five years have cleared the ground "for a final comprehensive attack," and they believe that "a definite solution of the problem is now in sight." Their product appears to be identical with Karrer's except for a possible discrepancy concerning the band at 693 mμ; but they think there is evidence of a possible contamination of the vitamin preparation with a foreign alcohol more saturated than the vitamin itself (see below).

Characterisation and chemical properties of vitamin A.—The viscous yellow oil, with molecular weight 320, and having the properties of an alcoholic derivative of carotene, obtained by Karrer, Morf & Schöpp, (1), was found to distil in a high vacuum with partial decomposition. It was reducible with loss of activity by Al-amalgam in ether, but resisted catalytic reduction. Oxidation with ozone yielded geronic acid, indicating the presence of the β-ionone linkage, and oxidation with KMnO₄ yielded acetic acid. The coloration with H₂SO₄ resembled that given by dihydrocrocetin, and the presence of six double bonds was thought probable (later measurements of the absorption maxima of carotenoids and vitamin-A concentrates with SbCl₃ are thought to indicate five double bonds: H. von Euler, Karrer, Klussmann & Morf). In the second paper by Karrer, Morf & Schöpp it is shown that the vitamin is an alcohol which forms esters with *p*-nitrobenzoic acid and acetic acid, from which it can be regenerated by saponification.

The formula proposed by Karrer⁵ is supported by the work of the Liverpool school. It is shown that degradation products can be obtained from vitamin A possessing the structure of naphthalene

⁵ Cf. *Ann. Rev. Biochem.*, 1, 557 (1932), for vitamin-A formula.

derivatives and having characteristic narrow absorption bands, formed presumably by ring closure of the vitamin-A molecule (Edisbury, Gillam, Heilbron & Morton; Heilbron, Morton & Webster). Karrer's conclusions are also confirmed in respect to elementary analysis, molecular weight, hydrogenation data, iodine values, ozonisation, and absorption spectra; and it is concluded that the preparations obtained by Karrer and by the English workers are in almost all respects "qualitatively and quantitatively indistinguishable" (Heilbron, Heslop, Morton, Webster, Rea & Drummond). There were, however, small discrepancies between the calculated and observed molecular weight, and inexplicable variations in intensity of the $SbCl_3$ absorption band at 693 m μ ; and hydrogenation experiments gave evidence of some contaminant being present.

Stability of vitamin A.—Dann, (1), has shown that there is an astonishing variation in the resistance of vitamin A towards oxidation by aeration, depending on the nature of the solvent in which it is held. The vitamin was particularly stable in ethyl alcohol, alcoholic KOH, and in ethyl acetate. R. J. Norris has measured the rate of destruction of the vitamin by ultra-violet irradiation, using biological tests. There was a one-hour induction period, after which destruction took place rapidly, being complete in eight hours.

Assay of vitamin A: (a) Biological method.—Several critical reviews have appeared discussing animal technique. Gudjónsson describes at great length his experiences with over 2,000 rats, and stresses *inter alia* the importance of standardised feeding of the mothers, and of the necessity of continuing the feeding of the curative dose for at least from eight to ten weeks. Coward deals with the "statistics of rat growth," based on 108 tests on 1,307 rats. She shows that twice as many females are needed as males to get a result of the same degree of accuracy: the steeper growth-dosage curve of the male more than compensates for his greater standard deviation. Nelson & Swanson discuss their results on 469 rats. The quantity of diet ingested was the principal variable, and the nature of the fat in the basal diet was of possible consequence.

Assay of vitamin A: (b) Chemical methods.—A number of modifications have been proposed for the $SbCl_3$ colour test. Morton adds 7-methyl indole with the purpose of inhibiting the 606 m μ band and measures the intensity of the 583 m μ band. Morgan suggests that certain irregularities in this test are overcome if the *blue-minus-yellow* value is taken, instead of the usual *blue* value, and this pro-

cedure is still satisfactory when applied direct to the original oil instead of to the unsaponifiable fraction. Mees finds that a 10 per cent solution of $SbCl_3$ in C_6H_6 is more stable, if somewhat less sensitive than the original Carr-Price reagent.

Moore's extraction method is accurate to 6-7 per cent, according to Simmonet, Busson & Asselin, and Norris & Church's technique for the assay is said to permit an error of less than 10 per cent (Dubin & Hooper). B. von Euler & Karrer likewise find the error to be no more than 10 per cent when working with cod-liver oils.

The apparent discrepancies between the two $SbCl_3$ absorption bands are further discussed by Ender; Lovern, Creed & Morton; H. von Euler & Karrer, (2); R. J. Norris; and Morton. The latter now leaves it an open question whether they may be due to two independent chromogens or to the complex colour mechanism of a single substance.

Coward, Dyer & Morton have continued their investigations on the relative value of the different available chemical and biological methods.

Occurrence of vitamin A.—Some seventy papers have appeared on this question, in addition to those dealing with the distribution of carotene.

(a) *Fish oils.*—Halibut-liver oil is now recognised as the richest known natural source of vitamins A and D. Emmett, Bird, Nielsen & Cannon assayed sixteen samples and found the vitamin-A potency to vary from 75 to 125 times that of a standard cod-liver oil. The considerable fluctuations in the activity of this material are of much commercial importance, but the causes are not yet properly understood (Lovern). Burbot-liver oil is another excellent source: it meets the requirements of the *United States Pharmacopoeia* for cod-liver oil (Braniion), and commercial specimens have been shown to be from four to ten times as potent as the latter (Nelson, Tolle & Jamieson). Many varied fish-body oils and their products have been discussed by other authors.

(b) *Other animal sources.*—As a general rule mammalian livers tend to be lower than fish livers in vitamin-D content, but they may often contain much vitamin A. Such has been shown to be the case for the blue whale, *Balaenoptera musculus* (Schmidt-Nielsen & Schmidt-Nielsen). Generally, of course, the activity depends largely on the vitamin intake in the food (work on vitamin reserves and storage has been discussed above), but Karrer, Euler & Schöpp say

that, surprisingly enough, little could be found in the livers of several species, *Felis leo*, *Felis tigris*, *Phoca vitulina*, although these had been fed largely on fish. As a general rule fish livers were more active than those of birds or of reptiles, which in turn were better than those of mammals. The highest value found was for *Rhombus maximus*, having 9,800 times the potency of cod-liver oil.

(c) *Milk*.—The influence of the diet of the lactating female upon the vitamin-A content of her milk has already been referred to. MacLeod, Brodie & MacLoon found from 1.3 to 2.0 rat units per gram in milk produced under excellent conditions of stall feeding, and there was little seasonal variation. Similarly, Crawford, Perry & Zilva found that butter from New Zealand, where, however, the cows are kept out at pasture throughout the year, varied but little in potency with the season. Neither is there significant variation between the milks of different breeds of cows—Holstein, Ayrshire, Jersey, or Guernsey (Davis & Hathaway). Ewes, asses, cows, and humans give milk containing similar amounts of vitamin A, according to Faberi & Sandicchi.

The soured-milk preparations, yoghurt and saya (but not kefir), are said to contain more vitamin A than the original milk, pointing to a synthesis by micro-organisms (Forster).

(d) *Plant sources*.—The richness in vitamin A of several plant sources has been stressed. The mango is equivalent to butter; the "Alphonso" variety was superior to two others tested (Perry & Zilva). The seed of *Bixa orellana* is one of the most active known sources, although, as shown by Euler, the pigment bixin itself is inactive (Cook & Axtmayer). In general, it may be said that green-leaved or yellow-rooted vegetables are excellent sources of vitamin-A activity and relatively stable to cooking processes (Scheunert). On the whole, rapid cooking appears to be less destructive of the vitamin-A content of foods than drying processes or prolonged storage (see, e.g., a paper by Newton).

VITAMIN D

A valuable addition to the literature of rickets is the comprehensive review by Goldblatt which has recently appeared. It contains no fewer than 2,723 references.

Incidence of rickets.—An illuminating statistical study by Maddox on 218 children at Sydney, Australia, showed past or present rickets in 52 per cent, active rickets in 12 per cent, healed rickets in

31 per cent, and doubtful in 7 per cent. In infants, over 33 per cent had active rickets, and it could be shown that the diet was deficient in vitamin D in 40 per cent of the cases. Experiments by Carstens in Holland show once again that rickets is almost inevitable in northern climates if suitable preventive measures are not taken.

Conclusive evidence has been brought forward to show that in India shortage of Ca and P from the diet may be as serious a factor as vitamin-D deficiency in the causation of rickets and osteomalacia [Wilson, (1); Hughes; Hughes, *et al.*]. Maxwell, Hu & Turnbull have published an important account of foetal rickets, attributed to the faulty diet of the mother.

Treatment of rickets and vitamin-D therapy.—A new natural antirachitic medicament is halibut-liver oil, which has some ten times the vitamin-D activity of cod-liver oil. About fifty papers published during the year have discussed the relative merits of various alternative methods of antirachitic treatment. The advantages of irradiated ergosterol therapy are illustrated by Schaferstein, who had unvarying success in a series of 315 prophylactic and 225 curative cases. In the United States legal action has been taken in a number of instances where medicinal products were found to have an insufficiently high biological value (Nelson & Walker).

To avoid hypervitaminosis, a dose of about 5,000 international units per day should not be exceeded [Harris, (2); E. Mellanby]. A collection has been made of the known instances of clinical hypervitaminosis [Harris, (2)]; the symptoms are identical with those seen in experimental animals.

Similar conclusions to those reached by Griffith, with the present writer, that vitamin D, although it aids calcification, does not retard tuberculous disease have been reached by Kaminsky & Davidson; Walker & Spies; Grayzell, Shear & Kramer; Crimm; Simmonet & Tanret; Arloing, Josserand, Ponthus & Vidailhet; Schaferstein.

Rickets in various species.—The occurrence of rickets in young turkeys is described by Scott, Hughes & Loy, in monkeys by Hottigner & Nohlen, in goats by Catel & Pallaske, and of tetany in pigs by Sheehy. Nitschke makes the very interesting claim that hedgehogs cease to hibernate when given vitamin D. Vitamin-D deficiency and the vitamin-D needs of chicks and hens have been discussed by some thirteen authors; of calves and cattle by about twelve writers; and of pigs by a number of others. Frateur says that one-third of the mortality of cattle in Brabant is ascribable to insufficient vitamin D.

Explanation of "anti-calcifying" influence of cereals.—An important note by Bruce & Callow discusses the so-called "toxamin factor" (Mellanby) of cereals. It is pointed out that the phosphorus of cereals is present mainly in the form of inositol phosphoric acid (phytic acid), which is not easily absorbed, but on hydrolysis by acid yields assimilable phosphate. This observation would appear to account for the "anti-calcifying" action of cereals, their phosphorus being in a condition unavailable for the needs of the body.

Vitamin-D action and hypervitaminosis.—The present writer has published a summarised account of experiments by himself and collaborators on the effects of large doses of vitamin D, and discussed these in relation to the light they throw on the mode of action of the vitamin. In the first place it was pointed out that the effects of moderate overdosage had to be distinguished from those of maximum toxic overdoses. With the latter, certain disturbing factors and quite distinct secondary effects are seen. Moderate overdosage, it is emphasised, leads to a fall in the faecal phosphate or calcium or both, to a rise in the blood phosphate and/or calcium, and to overcalcification at the growing end of bone. The latter is regarded as following mechanically from the increased $\text{Ca} \times \text{P}$ of the blood and taking place under the action of phosphatase, the "bone," or "calcifying," enzyme. It was pointed out that those soft tissues which become calcified in hypervitaminosis—the aorta, kidney, and intestine—are also richly provided with phosphatase, an observation which brings this phenomenon very logically into line with the general theory [see also Kay; Robison; Harris, (3)]. In short, just as deficiency of vitamin D causes increased faecal loss of P and/or Ca, a low blood P and/or Ca, and a resulting inadequate calcification, so on the other hand moderate excess of the vitamin causes a decreased faecal loss, raised blood P and/or Ca, and resulting excessive calcification. The observation that vitamin D gives rise to a diminution in the faecal excretion of phosphate and calcium can only mean either (a) that more is being absorbed from the gut, or (b) that less is being excreted back into the gut. Present knowledge does not permit one to decide definitely which of these two is the principal contributing factor. This was expressed by saying that vitamin D causes an increase in "net-absorption" (that is, "amount absorbed from gut less amount excreted back into gut"). The importance of the second of these two factors, i.e., excretion into the gut, has recently been stressed again by Taylor & Weld and by Shelling, but

these authors make the mistake of supposing that Harris and co-workers used the phrase "net-absorption" as synonymous with "absorption."

When maximum toxic overdoses of vitamin D are given, the extra calcium and phosphate drawn into the blood may now be derived largely from the bony storehouse, so that, although the epiphysis is still overcalcified, there may actually be a net loss of mineral matter to the bone as a whole. Furthermore, in the final stages of severe hypervitaminosis, gut function appears to break down and little, instead of more, Ca and P are now absorbed; but this may be largely the complicating effect of the general ill health of the animal and loss of appetite, since a similar result is seen in starvation or, in fact, in any severe indisposition. Be this as it may, outstanding features of experimental hypervitaminosis, which characterise the action of vitamin D, at whatever level it be fed, are: (a) the increase in blood P and/or Ca, first noted by Harris & Stewart, and (b) the overcalcification of growing bone recorded by Harris & Innes. (It may be recalled in passing that Kreitmair & Moll, who were the first to observe empirically that large doses of vitamin D were toxic, had not been able to give any rational explanation of the phenomenon, contenting themselves with the suggestion that the loss of weight denoted an increased basal metabolism or that the lesions resembled those of cholesterol arterio-sclerosis.)

The foregoing theory of vitamin-D action, it was shown, enabled one to account for a large range of observed facts—e.g., that the severity of hypervitaminosis depends on the Ca intake [Harris, (1)]; that more severe measures are needed to induce rickets in rats than in dogs or humans; that a high Ca/P ratio in the diet produces "low-phosphate rickets," and a low ratio low-calcium rickets and tetany; etc.

In many papers published during the past year it is still maintained that the toxic effects of irradiated ergosterol are due to some hypothetical independent toxic factor and not to vitamin D.* This view was strongly advocated by Dixon & Hoyle in England, and by Holtz and by Windaus and their co-workers in Germany. Harris & Moore, on the other side, advanced evidence that toxicity was an

* For various conclusions on this question see current communications by Goebel; Popoviciu; Popoviciu & Nitzescu; Nitzescu & Popoviciu; Kisch & Reiter; Laquer; Bomskov & Rose; Göttche & Kellner; Tixier; Bischoff & Loeschke; Windaus, Busse & Weidlich; Windaus, Lüttringhaus & Busse.

inherent property of vitamin D itself when fed in excess. The latter claim has now been finally vindicated by the proof of the toxicity of the pure crystalline vitamin D, calciferol (Askew *et al.*; Windaus, Busse & Weidlich).

Vitamin D and the parathyroid.—The action of the parathyroid resembles that of vitamin D in that it gives rise to hypercalcaemia, to calcium deposits in soft tissues, and to an increased urinary excretion of calcium (the latter symptom of hypervitaminosis was recorded by Harris & Moore in 1929). It has been maintained by many workers on these grounds that vitamin D functions by stimulating the parathyroids. In support of this theory it is also pointed out that administration of vitamin D helps to alleviate the ill effects of parathyroidectomy. As has been pointed out by Harris, (2), however, the action of the parathyroid appears to be normally quite independent of that of vitamin D. The parathyroid hormone raises the blood Ca solely by withdrawing Ca from the bones, whereas the characteristic action of the vitamin is to improve the utilisation of the food calcium, or, rather, to increase "net-absorption." Parathyroid, in contrast with vitamin D, raises the blood Ca only by draining calcium from the body. This difference is of practical significance in clinical medicine (see editorial, *Clinical Journal*).

Vitamin D and calcium metabolism in relation to dental disease.—Work published during the past year brings additional support to the view that deficiency of vitamin D is a factor conducive to dental caries. At the same time, evidence is growing of other factors which are of considerable importance. It will be recalled that past work by Mrs. M. Mellanby showed that those teeth which decayed were nearly always hypoplastic, while it was already well known that rickets may be a cause of one type of hypoplasia and also that caries was often particularly prevalent among victims of rickets. Following up these clues, clinical trials by M. Mellanby and co-workers showed that administration of vitamin D somewhat reduced the rate of spread of caries in children, although it by no means completely arrested it. Mrs. Mellanby's views, based on these observations, were recently summarised at a meeting of the British Medical Association. Arguments in opposition were arrayed by E. W. Fish, who maintained: (a) that the dental faults which give rise to caries are dissimilar from those resulting from rickets, and (b) that caries seemed too prevalent to be due to vitamin-D deficiency. The latter argument is perhaps unconvincing in the light of numerous recent surveys on the incidence

of rickets in many parts of the world. In the meantime Mellanby & Pattison have continued their clinical tests. By giving 5½-year-old children a diet devoid of carbohydrate and rich in vitamin D and in calcium, they have arrested caries to a somewhat greater extent than in their earlier experiments, where carbohydrates were not thus withheld. (This is attributed to the decalcifying ["toxamin"] action of the cereals, rather than to any local fermentative action in the mouth.) In apparent conformity with Mellanby's views is the fascinating report of Sampson, pointing out that the inhabitants of Tristan de Cunha, who enjoy an enviable freedom from dental disease, live on a cereal-free regimen consisting essentially of potatoes and fish. Another island community, in Pitcairn, live under similar conditions but substitute, in place of the potato, the yam, the sweet potato, and sugar cane, and have deplorably bad teeth (Sprawson). A possible weakness in this type of argument is that other variables, such as heredity, may be contributing factors. Thus it is difficult to account for the extreme prevalence of caries, e.g., among the Chiang tribesmen on the Tibetan border, whose diet is deficient in vitamins A and D and contains excessive carbohydrate but who live exposed to bright sunlight (Agnew & Agnew) or, for that matter, among the inhabitants of many parts of the tropics (Given).

An apparent difficulty in the way of the vitamin-D theory is the observation of MacKay & Rose, who found caries to be no more prevalent among a large group of children who were known to have had rickets in infancy than among normal controls who had not had rickets, although hypoplasia certainly was more prevalent in the former. MacKay & Rose seem quite justified from this experimental result in drawing the deduction that "vitamin-D deficiency in early childhood cannot be the main cause of caries." On the other hand, it has to be remembered that the children who had suffered from rickets were presumably treated with vitamin D and therefore eventually had more of it than their non-rickety controls, so that these results throw no light on the effect on the teeth of the dietary conditions of later childhood. And one has to bear in mind that statistics have been available since the middle of the last century showing that an apparent correlation often does exist between rickets and caries. In a paper published recently, Hjärne mentions incidentally the increased severity of caries among children having a history of tetany.

It has long been held that the carbohydrate or sugar in the diet favoured caries, not in any metabolic sense, but as a result of local

fermentation and saprophytic action on the tooth surface ("faulty oral hygiene"). The solution to the problem may very well turn out to be a combination of these two views, viz., that decay is due ultimately to faults in tooth structure caused by malnutrition (including vitamin-D deficiency among other factors), but more immediately to local fermentative action at the site of the fault. Shibata claims that easily fermented sugars in rats' diets inevitably give rise to caries.

A principal difficulty in elucidating this problem in the past has been the difficulty of producing caries experimentally. This difficulty now seems on the point of being solved, although many conflicting results are reported. Klein & McCollum make the definite claim that caries can be produced in rats as a consequence of persistent hypophosphataemia, brought about, of course, by means of abnormalities in the Ca/P ratio of the diet and vitamin-D deficiency. Dental abnormalities, chemical or morphological, in rats or dogs suffering from errors of calcium metabolism are reported also by Blackberg & Berke; Becks & Ryder; Karshan; Karshan & Rosebury (in the last case, on high-P, low-Ca diets, cf. Klein & McCollum; see also Forbes). On the other hand, negative results, which, however, are always of less consequence than a single convincing positive experiment, are reported by Lilly and also by Downs.

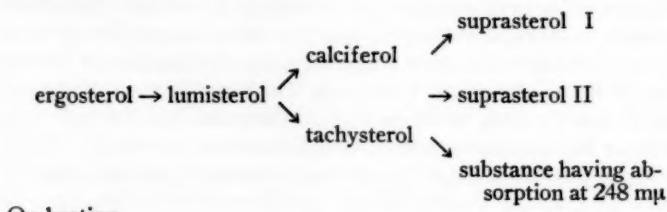
Striking clinical successes for dietetic treatment are recorded by Hanke. At Mooseheart, Illinois, 422 children were given 16 oz. of orange juice and one lemon each per day. It is said that all gingivitis not associated with calculus rapidly disappeared, and caries was arrested in 49.8 per cent of the cases. It is not certain whether this was due to the vitamin C of the fruit juice, or other constituents (? phosphate).

Isolation of vitamin D.—The fuller details have since appeared of the procedure used for the isolation of vitamin D, reported in Volume I, i.e., through fractionation as 3,5-dinitrobenzoate by the Mount Vernon workers (Askew, Bourdillon, Bruce, Callow, Philpot & Webster); or by means of the maleic or citraconic anhydride process of Windaus *et al.* The English workers admit the identity of their "calciferol" with the "vitamin D₂" of Windaus. Among other matters they discuss the properties of two allied substances, pyrocalciferol (formed from calciferol by heat) and sterol X (= lumisterol). The vitamin D₁ of Windaus was shown to be a molecular compound of calciferol and lumisterol (see also Windaus, Ditmar & Fernholz).

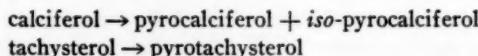
That calciferol is a single substance can be regarded as virtually certain from the X-ray examination by Bernal.

It is stated that a purely chemical activation of vitamin D, as distinct from photochemical, has been achieved by Bills & MacDonald by warming ergosterol with methyl alcohol, ether, and ethyl acetate; but details are not yet available. Windaus, Linsert, Lüttringhaus & Weidlich have measured the melting-points of calciferol and various derivatives, their rotations in different solvents, etc. These constants agree excellently with those quoted by the English workers.

Photochemistry of vitamin D.—The nature, number, and order of formation of the various irradiation products of ergosterol have been studied by Windaus, Lüttringhaus & Busse. Their conclusions may be summarised thus:



On heating,



Tachysterol is readily oxidisable, and its presence is supposed to induce destruction of the vitamin (see also Inhoffen & Hauptmann). It is credited with toxic properties, like several other of these by-products, although it has not yet been isolated and tested individually.

Earlier work had shown that vitamin D can be activated not only by ultra-violet rays but also by X-rays or cathode rays. Askew, Bourdillon & Webster have now demonstrated that a glow discharge will also serve. Vitamin D has absorption bands in the infra-red as well as the ultra-violet, and is destroyed by infra-red rays (Hirsch & Kellner).

Vitamin-D occurrence.—About sixty papers dealing with the distribution and occurrence of vitamin D have appeared during the period under review. The most striking recent advance is the discovery that halibut-liver oil is richer in vitamin D (and A) than any natural source hitherto known (see Emmett, Bird, Nielsen & Can-

non). Commercial specimens of burbot oil had from three to four times the activity of good cod-liver oil (Nelson, Tolle & Jamieson).

Irradiated milk, dairy produce, etc.—The effect of irradiated ergosterol fed to cows upon the antirachitic potency of their milk is well illustrated by the following figures (Krauss, Bethke & Monroe):

Butter from control cows.....	0.17 units per gm.
Butter from cows fed 200,000 units of vitamin D.....	2.5 units per gm.

Clinical trials of irradiated milk (Gerstenberger & Horesh) demonstrate again that it is too mild an antirachitic agent to be suitable for curative treatment, whatever may be its use in prophylaxis.

VITAMIN E

The romantic suggestion that "royal jelly," the consumption of which, it will be recalled, converts the larva of a working bee into a queen, owes its property to the presence of vitamin E is made by Sir Leonard Hill & E. F. Burdett. In support of this idea they present the experimental evidence that the feeding of "royal jelly" to three rats for one month "effected the production of two full-term litters." No full details have yet appeared, however, of an assay by the standard technique.

The concentration of vitamin E has been carried a little further by Olcott, who, starting with a concentrate prepared from lettuce by the Evans-Burr method, obtained a fraction of increased activity by distilling it *in vacuo* at 190°-220° (0.1 mm.). By then removing sterols and solid alcohols by crystallisation from acetone, a preparation active in doses of 10 mg. was gained. Acetylation and hydrogenation did not affect its activity.

So far the evidence has been sparse of any practical correlation between vitamin E and sterility in human beings. Vogt-Möller has now published in the *Lancet* a record of two women who, after four or five consecutive miscarriages, each bore a live child upon treatment with vitamin E. Much additional controlled data will, needless to say, be needed before any definite conclusions can be reached on this aspect of the vitamin-E problem.

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NUTRITION*

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The purpose of the *Annual Review* is to chronicle the yearly contributions to the expanding science of biochemistry. The need for this enterprise is particularly pressing in the field of nutrition where the current progress is unusually rapid. The separation of the subject into topics representing still further specialization, as adopted by the editors, is evidence that they are cognizant of the large scope of that part of biochemistry included in nutrition. Perhaps no better manifestation of the interest in this subject can be cited than to point out that within the past two years there have appeared two new journals dealing more or less specifically with nutrition, namely, the *Zeitschrift für Ernährung* and the *Nutrition Abstracts and Reviews*.

The changes in nutrition which inevitably accompany national development have been emphasized by Mendel (1) in connection with the diet of the American people. The marked simplification in menus is pointed out as is also the unmistakable decrease in consumption of cereal foods and meat. Furthermore, no doubt is left that the discoveries in connection with vitamins and deficiency diseases have also had an important influence upon the changing character of our diet.

Nutritional edema among human individuals attracted considerable attention during the World War, and in the intervening years experimental studies have demonstrated the correlation between the disturbed fluid balance and the concentration of serum proteins. Ling (2) has reported a lowered level of serum albumin in the blood of undernourished Chinese, accompanied by edema, negative nitrogen balance, and a subnormal basal metabolic rate. Administration of a protein-rich ration produced a prompt subsidence of the edema but only a slow return to the normal level of serum proteins. The significance of the amount and quality of dietary protein is emphasized by Liu and co-workers (3) who were able to accentuate the edema and to lower the serum proteins in two patients by means of

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a nitrogen-free diet. When 7 per cent of the food energy was given as animal protein (1 gm. per kilo per day) a response both in the edema and in the serum protein concentration was observed; with vegetable protein, however, twice the amount was necessary to produce a similar effect.

An interesting instance of endemic edema of nutritional origin was reported by Youmans (4) in this country. It occurred most frequently in women, was most pronounced in spring and early summer, and was unusual in that the serum protein of those affected was little below the normal values in concentration. Most of the individuals studied had used the low-protein dietary throughout life as a matter of custom rather than one of financial necessity.

Experimental studies have been carried out which have a bearing on the mechanism of nutritional edema. Using dogs maintained on a low-protein diet, Lepore (5) produced edema by removing blood and returning to the circulation the washed blood cells and Locke's solution. Edema occurred when the plasma protein concentration dropped below 4 per cent and was associated with retention of sodium chloride, largely in the muscle, and of water, principally in the skin. Similar experiments by Barnett, Jones & Cohen (6) led to the conclusion that loss of plasma proteins was not alone the cause of the diminished concentration of these constituents in the circulating blood. Large quantities of plasma protein were withdrawn—as much as 7 gm. daily—without producing a change in the level of plasma protein concentration. However, a liberal diet of meat was provided which, in itself, is one point of difference from the usual dietary conditions associated with nutritional edema.

The results of an investigation designed to bring out the influence of nutritive state on behavior were reported by Anderson & Smith (7). One group of albino rats whose growth had been inhibited by a lack of lysine in the diet and another, restricted by underfeeding with an adequate diet, were compared to normal control animals on the basis of measured activity, behavior in mazes, and in a problem box. At all ages the stunted animals were more active and learned the maze problems more quickly than the controls, although they were less accurate. In all groups speed decreased with age. The rats restricted by lack of lysine were most adept at solving the problem box, and in all nutritional groups the older animals required less time to master this test than did the younger ones.

PROTEINS IN NUTRITION

The question as to the reliability of instinct in selecting an adequate diet has again been studied. In experiments of Kon (8) young rats were given a choice of a salt mixture, casein and carbohydrate (sucrose or rice starch) together with the necessary vitamins. It was found that, over a period of 69 days, they consumed only 6.5 per cent protein compared to 20 per cent for the controls on a mixed diet. The growth of the rats with free choice was less rapid than that of the controls. It appears that, under the experimental conditions established, the low intake of protein was the factor limiting the development of the experimental animals.

The wide use of animal concentrates rich in protein for nutrition investigations lends significance to a study of the nutritive value of these substances reported by Curtis, Hauge & Kraybill (9). It was emphasized that the total nitrogen of meat scrap, stick, tankage, and similar products is a poor index to the content of physiologically available protein and that the type of animal refuse going to make up the concentrate must be considered in evaluating it for use in experimental rations. Another study bearing upon technique deals with the determination of the biological value of protein in birds. St. John, Johnson, Carver & Moore (10) calculated from existing data that 80 per cent of the total urinary nitrogen in birds is made up of uric acid and ammonia nitrogen and employed this factor for estimating that part of the excreted nitrogen which can be designated as fecal. They found that, in chickens over a wide range of ages, the metabolic fecal nitrogen was 2.85 gm. per kilo of food and that the endogenous nitrogen of the urine was 0.405 gm. per kilo of body weight.

The protein requirement for optimal growth as well as practical feeding problems involving supplementary action of proteins has been studied. Milne (11) came to the conclusion that, for growing chicks, optimal development takes place with 30 to 55 per cent protein in the diet. With levels of 5 per cent or 80 per cent, growth was not favorable. However, the point of view that mineral constituents rather than the proteins are the prime factors in rapid growth in chicks is emphasized by Prentice & Baskett (12). They assert that, in the presence of an adequate supply of salts, little difference is observed between vegetable and animal proteins as growth-promoters.

The traditional adverse attitude of students of nutrition toward

gelatin is again given support by its failure to improve the nutritive value of potato protein [see Jones & Nelson (13)]. Gelatin alone at a concentration of 20 per cent or when supplemented with cystine, tyrosine, and tryptophane caused no improvement in growth, although casein or lactalbumin, similarly used, promoted normal growth. Inasmuch as yeast and casein have proved to be convenient sources of protein for experimental rations, Shrewsbury, Vestal & Hauge (14) have investigated the possibility of using these food materials on a commercial scale. Growth of rats and of pigs was improved by including 2.25 per cent to 5.0 per cent casein in a basal diet of corn, soybeans, and minerals. As much as 3.0 per cent of dried yeast failed to enhance the nutritive value of the basal diet sufficiently to be of economic value. The nutritive deficiency of soybeans, even after being cooked, is emphasized in this study. That irradiation of protein improves its nutritive value is suggested by experiments of Koch & Koch (15), in which better growth was secured when casein was irradiated. However, the basal ration contained no vitamin D other than that in the butter fat, and the enhanced growth may well have resulted from the added quantity of this factor produced by irradiating the casein. This conclusion is supported by the unchanged retention of nitrogen after irradiation.

Recent contributions have been made to the subject of the amino acids essential in nutrition. Rose (16) has summarized the older literature and also has discussed his own studies, which lead to the conclusion that in the monamino fraction of hydrolyzed casein there is present a hitherto unrecognized dietary factor required for growth. These investigations have been extended by St. Julian & Rose (17) who have indicated that neither the dicarboxylic acids, proline and oxyproline, nor arginine, aspartic acid, and the glutamic acids are indispensable in the diet.

Bing, Adams & Bowman (18) have reached the conclusion that the protein requirement of the mouse is not different from that of the rat. On rations composed of purified food substances the best growth response was observed when the protein (casein) calories constituted between 7.8 per cent and 15.6 per cent of the total energy intake.

An instance of maintenance upon an exceedingly low intake of protein was reported by McClellan & Hannon (19). The subject received 20 gm. of protein daily for 106 days in a diet providing 2,000 calories; body weight was maintained. On one day the excre-

tion of nitrogen reached the low level of 1.78 gm. It is significant that, although the ration contained a relatively small amount of carbohydrate, the excretion of nitrogen slowly diminished throughout the experimental period. Stored body fat may provide calories and the tissues be spared even in the face of marked deficiency of total energy. Keeton & Dickson (20) have reported that during the loss of weight in obese individuals, nitrogen equilibrium can be maintained when the food consumption is 30 to 50 per cent below basal requirements. According to Barker (21) fatty degeneration of the liver may occur in dogs on a ration deficient in protein but otherwise satisfactory. The resulting failure of appetite can be corrected by giving protein, but the repair of hepatic tissue is very slow.

The evidence for the influence of dietary protein upon certain organs, notably the arteries and kidneys, has been surveyed by Bischoff (22). Granting that tissues can be damaged by constituents of the diet, he questions whether the existing evidence justifies the conclusion that individual food components are able to produce pathological conditions closely resembling human nephritis or arterial sclerosis.

FATS IN NUTRITION

Recently Burr & Burr (23, 24) described experiments with albino rats which led them to conclude that, in animals given a ration extremely poor in fat, a deficiency disease results which can be cured by linoleic or linolenic acids or fats containing these acids. Wesson & Burr (25), on the basis of respiratory studies, attempted to determine whether or not in rats with the "fat disease" the body fat laid down after dextrin was given contained the indispensable fatty acids. The respiratory quotient indicated that fat was formed from the dextrin, but the failure to cure the disease convinced the investigators that the body fat thus formed did not contain the essential fatty acids. However, this observation does not necessarily constitute negative evidence for the thesis, inasmuch as the alleged efficacy of the unsaturated acids would lie rather in their activity in metabolism than as storage material. Support for the foregoing conception of the essential nature of the two fatty acids is contained in two reports by Evans & Lepkovsky (26). The tail necrosis was not observed in these studies, but failure to grow, malnutrition, hematuria, increased water intake, and response to small amounts of fat were used as criteria of the presence of the disease. Cures were

obtained with rice and corn starch, liquid fatty acids from cocoanut oil, linoleic acid, and the fatty acids from rice and corn starches. These authors also stated that growth would not take place unless unsaturated fatty acids with two or more double bonds were present in the ration. Burr, Burr & Miller (24) observed that tung oil, like butter, was somewhat effective in curing the syndrome produced in rats on a fat-poor ration and concluded that this oil contained undetermined fatty acids essential in nutrition. Further evidence in support of the contention of Burr & Burr (23) that the animal body cannot synthesize the essential fatty acids was provided by Evans & Lepkovsky (27) who observed that the condition resulting from maintenance on a fat-free diet was cured by feeding 5 drops of the methyl esters of the fatty acids obtained from the body fat of rats grown on a stock ration whereas the body fat of rats on a fat-free diet did not have the curative effect. It was pointed out that the efficacy of the fatty acids was not parallel to the iodine numbers. Sinclair (28) has likewise noted that the tissue phospholipids of rats on a fat-free diet have fatty acids with a normal iodine number, a situation difficult to explain on the basis of the indispensability of linoleic and linolenic acids.

MINERALS IN NUTRITION

Viewpoints in nutrition have undergone a definite change during the past twenty-five years. Not only have biochemical investigations unfolded hitherto unsuspected dietary factors which play a significant rôle in physiology, but quantitative studies have emphasized the potency of many of these by showing that mere traces of several substances, such as vitamins and certain mineral elements, suffice to promote normal function in the organism. Some of the newer facts have been treated by Rose (29) who points out the nutritional significance of iodine, fluorine, aluminum, zinc, copper, manganese, and iron. Thatcher (30), in discussing the newer aspects of the nutrition of plants, emphasizes the fact that, in addition to the demonstrated requirement for potassium, nitrogen, and phosphorus, plants must be supplied with traces of elements not hitherto thought to be important for optimal growth. Thus manganese, zinc, boron, and copper have been shown to be important in the development of certain farm-crop plants and vegetables.

Recent experiments have demonstrated that magnesium must be considered as an indispensable dietary constituent. When the content

of this element in the ration of rats falls below 30 mg. per 100 gm. of food, growth is subnormal according to Lavollay (31). Development at the normal rate was promoted by 55 mg. per 100 gm. ration. However, when this quantity of magnesium was provided in the basal diet employed by H. von Euler & Virgin (32) subnormal growth resulted, whereas maximal development was obtained with 150 mg. per 100 gm. of ration. Kruse, Orent & McCollum (33) reduced the magnesium content of their experimental diet to 1.8 γ per gram. After three to five days on this otherwise adequate diet, young rats showed striking vasodilatation, hyperirritability, spasticity, rapid heart beat, and arrhythmia, and succumbed early. There was no loss of appetite or of body weight. Generally similar symptoms were noted in dogs by Orent, Kruse & McCollum (34). Not only was vasodilatation observed but hemorrhage and convulsions followed by extreme nervous exhaustion. As little as 0.05 per cent magnesium prevented the syndrome, but it is not clear whether or not these protected animals could be considered normal. These authors (35) also observed in the blood an early and progressive diminution in magnesium, an increase in cholesterol esters shortly before death, and a fall in the hematocrit value. The bicarbonate concentration remained within normal limits and, although tetany was observed, the calcium level was not low. Excess (1.6 per cent) magnesium fed to rats on a diet of purified food substances produced urinary calculi in many cases, more frequently in females than in males [Watchorn (36)]. This lithiasis could be prevented by adding calcium carbonate to the diet.

From the foregoing discussion it is apparent that the recent contributions have emphasized a new point of view respecting the significance of magnesium in nutrition. Whereas previously its presence in the tissues and fluids of the body was taken more or less for granted and studies were mainly confined to the antagonism between calcium and magnesium in metabolism, at present it appears that at least a trace of magnesium in the diet is indispensable for the physiological well-being of the organism.

Following the conflicting reports of the part played by manganese in the cure of nutritional anemia in rats, biochemical interest has turned to the evaluation of this element as a stimulant of growth. One of the earliest of such studies was made by Nelson, Evvard & Sewell (37) on rats with a diet of purified food materials. The addition of 100 parts of manganese per million promoted better growth

than was shown by the control animals but 200 parts were apparently too much. Striking increases in appetite and growth were obtained by Skinner, Peterson & Steenbock (38) when as little as 4.1 γ of manganese per day was added to a milk-copper-iron ration given to young rats previously deprived of manganese. An acid extract of alfalfa had a similar influence. Kemmerer, Elvehjem, Hart & Fargo (39) have reported excellent growth in rats on fluid milk supplemented with 0.5 mg. of iron, 0.05 mg. of copper, and 0.04 mg. of manganese per 300 cc. of milk. Excellent growth was also obtained in young pigs maintained on this ration for sixteen weeks. In contrast to these observations are those of Orent & McCollum (40), who observed no influence on growth of rats after adding manganese either to a solid ration or to milk supplemented with iron and copper. A relatively high intake of manganese (2,000 parts per million) had no deleterious effect on the growth of young rats for the first twelve weeks after weaning [Skinner (41)].

Attempts have been made to improve reproduction in rats subsisting on a diet of fresh milk. Skinner, Van Donk & Steenbock (42) reported that the addition of manganese to milk already supplemented with iron and copper did not improve the abnormal estrous cycle in rats. However, when energy in the form of sugar was added, manganese did seem to exert a favorable effect. On the other hand, Orent & McCollum (40) failed to observe any abnormality in the estrous cycle in rats either on a solid ration or on a milk-iron-copper diet, whether or not manganese was added. Skinner (41) did not find a deleterious effect on reproduction when an excess of manganese (10 mg. per day) was fed.

The iron requirement for children has again received attention. Leichsenring & Flor (43) fed two levels of iron (3.25 mg. and 6.50 mg.) to four children and observed that there was a greater retention of iron on the higher intake. They concluded that 0.48 mg. per kilo of body weight, or 0.62 mg. for each 100 calories, would be a standard allowance for maintenance and growth in the pre-school child. Coons (44) studied the iron balance of nine women during pregnancy. The balances varied from 0.88 mg. to 6.99 mg. per day. In view of the considerable quantity of iron in the body of the newborn (*ca.* 375 mg.) it was pointed out that only with very favorable diets was enough iron provided for maternal needs over the requirement of the fetus. Meat, eggs, and green vegetables exerted a more favorable influence on iron retention than did other foods. On a

diet of cooked rice and raw milk providing 5.0 mg. of iron per day, Fontés & Thivolle (45) showed an average daily loss of iron of 4.85, 5.86, 6.19, and 14.25 mg. in each of four dogs respectively over a period of six weeks. In twelve dogs subjected to experimental hemorrhage at intervals of two weeks, these authors (46) observed an average positive balance of 2.3 mg. after the third bleeding.

Drinking water with a high salt content may exert a retarding effect upon growth. Heller (47) made this observation in young rats and also reported an interference with reproduction under like nutritional adjustment. These effects seem to be due to osmotic influences rather than to specific ions.

NUTRITIONAL ANEMIA

Mitchell (48) has summarized some of the necessary precautions in conducting experiments in this field. The hemoglobin of the young is only slightly influenced by the composition of the diet of the mother and not at all by the pigment content of her blood, by her age, or by her weight. Females have a greater concentration of hemoglobin than do males, a fact which, along with the prevention of coprophagy, influences the speed of production of the anemia. The theoretical value of glass cages in such studies has been questioned by Skinner, Steenbock & Peterson (49) who state that the inevitable soiling of the sides and bottoms is an interfering factor in studies of anemia. This observation has also been made by the reviewer in studies involving strict limitation of mineral salts.

The type of milk best suited to produce anemia has been discussed. Harris (50) advocated the use of whole milk powder (Klim) as the basal ration; his analyses showed 0.00024 per cent of iron and too little copper to give a color with pyridine, carbon tetrachloride, and thiocyanate. Natural milk, the same fresh milk after being boiled for two minutes, and reconstituted dried milk with the same iron and copper content as fresh milk are equally suited for the production of anemia according to Supplee, Dow, Flanigan & Kahlenberg (51). On the other hand, roller-process dried milk with a high iron content not only will not produce anemia but also will cure it better than the addition of iron and copper. Krauss, Erb & Washburn (52) were able to render young rats anemic with milk both before and after pasteurization.

The failure of iron alone, glutamic acid, tyrosine, tryptophane, aspartic acid, or arginine to cure milk anemia has been reported by Keil & Nelson (53). They maintain that copper alone is effective in the ability to supplement iron in this connection. However, ferric citrate given intraperitoneally gave an increase in hemoglobin, and hydrochloric acid similarly administered resulted in a slight temporary response. Elvehjem & Hart (54) observed striking increases in hemoglobin concentration in pigs on a milk diet when copper (5.0 mg.) was given along with iron (25 mg.), although iron alone had no such influence. The failure of iron alone, of iron and manganese, and of a mixture of manganese, cobalt, nickel, zinc, and iron to cure anemia was demonstrated by Orten, Underhill & Lewis (55) whereas under similar conditions copper was effective. A contribution of considerable interest in this general connection is the report of a spectrographic examination of a large number of therapeutic iron preparations by Sheldon & Ramage (56). Manganese was a constant impurity in the entire number (65) of substances studied. Copper was absent from only 20 per cent of the preparations. It appears that the efficacy of large doses of such substances might well vary according to the contamination, inasmuch as the supplements are required in mere traces.

Is iron in organic combination as effective with copper as is inorganic iron? Elvehjem (57) has shown that both ferric chloride and hematin are without value in the absence of copper but that when copper is given the response with inorganic iron is far superior to that with hematin. It appears that the iron from hematin is less readily absorbed than that from ferric chloride.

The erythropoietic effect of cobalt has been studied further by Orten, Underhill, Mugrage & Lewis (58). When cobalt, iron, and copper were added to fresh milk, the red cells increased from the normal value of 8,300,000 to 12,200,000, and the hemoglobin from 13.2 gm. to 18.3 gm. This polycythemia was produced with 0.5 mg. of cobalt(ous) either as the chloride or the sulfate but without copper the response was not obtained. The existence of an unidentified organic component of food materials which is effective in stimulating the production of blood pigment is indicated by experiments of Rose & Vahlteich (59). Whole-wheat flour and oatmeal produced regeneration of blood pigment on a milk diet far better than could be accounted for by their content of iron alone. The ash of the oatmeal or of the whole-wheat flour did not stimulate hemoglobin

production like the equivalent amount of the food itself. In another paper Rose & Kung (60) evaluated whole wheat as a source of iron for hemoglobin regeneration in comparison with bran and liver. On the basis of a rather complicated experimental procedure, the conclusion was reached that liver was approximately half as effective as a source of iron as is whole wheat, which is also superior to bran in this respect. Levine, Culp & Anderson (61) concluded that the efficacy of dried vegetables, fed on the basis of the iron content, depends upon the amount of copper also contained in the material. Oysters are second only to liver as a cure for anemia according to Coulson, Levine & Remington (62), those from southern waters having more iron and less copper than those from the waters of the North Atlantic states. The extensive use of soybean products among the Chinese gives significance to the observation of Adolph & Kao (63) that milk anemia in rats is cured by soybean meal, soybean cheese, and soybean milk. Analyses showed that, as ordinarily used, these products contain appreciable quantities of iron and copper.

A suggestion as to the mechanism of the supplementary action of copper was made by Josephs (64). According to him, iron alone added to a diet increases the tissue iron but has no effect on hemoglobin iron, whereas addition of copper is followed by a reduction of tissue iron and an increase in the iron of the blood pigment. Copper does not increase the retention of total iron but is extremely effective in the transformation of stored or absorbed iron into hemoglobin. An essentially similar mechanism is postulated by Elvehjem & Sherman (65), who observed increases of iron in spleen and liver when iron alone was given to anemic rats. When iron was removed from the diet and copper given, the hepatic iron decreased, the hemoglobin increased, and the volume of the spleen increased though there was little change in its iron content. These investigators state that, when iron and copper are given together, little iron is stored in the liver until 0.3 mg. or more is given in the food. Evidence in support of this view is furnished by experimental data on two patients [Resnikoff (66)]. A normal individual stored 383 mg. iron over a period of seven months and a polycythemic patient treated with phenylhydrazine retained 852 mg. over a similar interval; in neither case, however, was the stored iron utilized for pigment formation. Boganiard & Whipple (67) have discussed the comparative activity of various organs in storing iron. They pointed out that the kidneys are important in conserving iron but that the spleen and, especially,

the liver are the main organs where iron is segregated; conversely they show marked diminution in iron after hemorrhage.

That pigment production can be stimulated apart from erythrocyte formation is indicated by Schultze (68) who found that nickel alone produced an increase in cells without greater formation of pigment and that copper had little effect on hemoglobin. Pigment formation is possible only when iron is available, whereas red cells can increase without iron. Although this is a reasonable hypothesis, some of the contributing experimental results of Schultze do not agree with accepted observations in this field. However, Stein & Lewis (69) have noted that rats on raw fresh milk and copper alone showed a low level of pigment with little reduction in number of red cells; the same results were secured with evaporated milk. The authors suggest that copper stimulates erythrocyte formation whereas iron is necessary for pigment production.

Swanson & Smith (70) have described a type of nutritional anemia not typical of that produced by feeding milk. With a solid ration consisting of purified food substances in which the total inorganic residue amounted to only 0.94 per cent, a diminished concentration of hemoglobin, an increase in the number of red cells, and a decrease in size of the individual erythrocytes developed progressively in young adult rats over a period of 90 days. Nevertheless, the concentration of pigment within the cells was not abnormal at this time.

Does nutritional anemia produced by exclusive feeding of milk to experimental animals have a counterpart in the clinic? This question will always be difficult to answer because of the limitations to proper control of conditions when working with human subjects. A beginning might well be made by following the suggestions of Osgood & Haskins (71) for reclassifying anemias in the light of the more recent contributions. Sherman (72) has employed essentially the same classification in the latest edition of his textbook.

That anemia in infancy is probably referable both to failure of storage of iron and copper at birth and to the deficiencies of the diet during the first six months of life is the opinion of Greengard (73). Liver and iron are considered by him as the ideal combination for combating this condition. Mackay & Goodfellow (74) recommend iron in dried milk for the anemia which they observed in both breast-fed and artificially-fed infants. They are not certain about the value of copper. Human anemia only rarely arises from dietary causes

alone according to Kern (75) and a relatively long time is required for a poor ration to bring about this result. In the experience of Maurer, Greengard & Kluver (76) copper and iron without other therapy failed to produce a remission of infantile anemia in 50 per cent of the cases, whereas subsequent addition of liver to the iron and copper cured the condition. Clark (77) concluded that only when the iron store is low is iron therapy efficacious and even here it is improbable that the beneficial effect is due to contaminating copper. Copper alone or along with iron was not a potent cure for anemia in premature infants in the experience of Schiff & Joffe (78). It is apparent that the available evidence does not at present permit the direct translation of experimental results to human experience in the matter of anemia. Nevertheless, dietotherapy has undoubtedly benefited from the observations made in connection with studies of nutritional anemia in experimental animals.

NUTRITION AND GROWTH

To what extent can the growth of animals be improved through a more favorable dietary environment? Unusually rapid increase in body weight was reported by Bryan & Gaiser (79), who, in addition to feeding a favorable ration, injected a preparation of pituitary hormones into rats. The average daily rate of gain of fifteen of the best animals between 60 and 200 gm. on the better diet was 6.1 gm. Anderson & Smith (80) have obtained extremely rapid growth with male rats on an adequate ration somewhat richer in protein than is usual in experimental feeding but without recourse to other growth stimulants. The average daily gain of twenty-one unselected animals from 60 to 200 gm. was 6.1 gm.; from 60 to 300 gm., 6.0 gm., and for nineteen rats from 60 to 400 gm., 5.2 gm.

Excellent records for reproduction and growth have been published by Wu, Wan & Chen (81). On a ration of whole wheat, millet, heated soybeans, beef, cod-liver oil, yeast, salts, and vegetables the males in the group grew from 60 to 200 gm. in 31 days and from 60 to 300 gm. in 63 days. When rats four months old were placed on a vegetarian diet the body weights at subsequent intervals were less and mortality among the young greater than on the same ration containing dried meat [Wan & Wu (82)]. Further evidence of the value of meat in growth and reproduction is furnished by Scheunert & Venus (83), who observed the same favorable results whether the meat was raw, fried, boiled, or frozen.

It is known that experimental animals grow at an accelerated rate after the diet is changed from one of poor quality to an adequate one. Stearns & Moore (84) have described a similar set of circumstances in a male child which, at 3½ years of age, weighed only 7.4 kilos and was 81 cm. long. It had suffered from severe malnutrition, diarrhea, and infections of ears and sinuses. On a ration rich in protein, low in fat, and with simple carbohydrates the weight had increased 11 kilos in nine months and the length 14.5 cm.; the normal gain during this interval for this age is 1.2 kilos and 4.0 cm.

Limson & Jackson (85) have made an extensive study of the structural changes in rats subsisting on a diet very low in protein. Growth of the body as a whole was inhibited, although, in common with results of other types of stunting, body- and tail-length increased. The weight changes in the organs varied, some increasing, others remaining unchanged, and still others decreasing. The suggestion was made that similarities in change produced by various types of malnutrition are due to a common underlying cause, whereas differences may represent specific effects.

The growth in volume of the entire body and of the various parts was determined in two groups of boys ranging from five to nineteen years of age by Zook (86). One group was poorly nourished and the other well nourished. The cranium and foot were the only parts growing alike in the two groups; the poorly nourished group had lesser absolute values for other parts of the body. There appeared a lag of relative growth in late childhood and an acceleration during adolescence in all parts except the foot and cranium.

NUTRITION AND THE REPRODUCTIVE CYCLE

The ratio of nutrients ingested in the food to those secreted in the milk was determined by Shukers, Macy, Nims, Donelson & Hunscher (87) in three human subjects. The ratios, expressed as percentages, were for calories 50, 33, and 30; for fat 40, 36, and 48; for carbohydrate 70, 70, and 68; for protein 10, 10, and 12; for calcium 41, 27, and 15, and for phosphorus 11, 11, and 6. These data emphasize not only the large proportion of nutrients which may be eliminated in the milk but also the importance of aiding the maternal organism to conserve these materials to the utmost. A similar study was carried out by Forbes & Voris (88) on nine cows over an average lactation period of 313 days. The average proportion of food energy appearing in the milk was 21 per cent.

The level of dietary protein is an important factor in maintaining a normal estrous cycle. Guibert & Goss (89) observed a failure of normal estrus in rats when the protein content of the diet was below 5 per cent. Failure to mate and death of embryos was also noted. The age when the protein was reduced had little effect on the disturbance. Reproduction and lactation on a whole-wheat and whole-milk diet was much enhanced by adding fresh beef or meat scrap [Russell (90)]. The improvement was not due to increase in quantity of calcium or phosphorus or to a better ratio but rather to some substance in the meat which is not contained in dried yeast. The estrous cycle is lengthened when rats are restricted to a vegetarian diet [Lin, Tsai & Wan (91)]; the di-estrous interval is prolonged, though the cornified cell stage is not affected.

That raw liver contains a factor exerting a favorable influence on lactation and growth has been observed by Smith (92). This effect is apparently not due to either the fat or the protein of the liver. The substance in liver which promotes lactation and stimulates growth is passed on to the young in the mother's milk [Mapson (93)]. It is not identical with recognized food substances, is precipitated from fresh liver by 90 per cent alcohol, and is most readily obtained by aqueous extraction of autolyzed liver.

The maintenance of a positive calcium and phosphorus balance during pregnancy is of great importance, inasmuch as the subsequent lactation is usually characterized by a negative balance, especially severe for calcium. Toverud & Toverud (94) found that 1.7 gm. and 1.8 gm. of calcium and phosphorus respectively are necessary to prevent a negative balance in human subjects during the last two months of pregnancy. In commenting upon the exaggerated losses of calcium and phosphorus during lactation Hart (95) pointed out that the normal mechanism for combating withdrawal of calcium in the milk is an increased activity of the parathyroid glands. Failure of this results in milk fever, and the study of Wilson & Hart (96) demonstrated that during this condition the levels of calcium and phosphorus in the blood are definitely reduced.

Pregnancy is commonly looked upon as a period when material is lost by the maternal organism. In extensive experiments Mitchell, Carroll, Hamilton & Hunt (97) have shown that in pregnant, growing hogs the available nutrients of the ration are accumulated in the maternal body at a faster rate than in the fetal body. After sixteen weeks the fetuses and adnexa accounted for only 31 per cent of the

live weight gained during that interval by the sow. In general, similar conclusions were reached by Agduhr (98), who showed that not only was reproduction in mice accompanied by increase in body- and organ-weights but also these increases were proportional to the number of pregnancies.

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THE CHEMISTRY OF MUSCLE*

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The achievements¹ of the biennium 1930-31, which have been referred to in the previous review,² have recently been fully considered by a number of authors [e.g., Hill (71, 72), Needham (110)]. Much of the newer work on muscle chemistry is concerned with the extension and consolidation of these achievements.³ In some new fields, however, considerable progress has been made. New physical phenomena of muscular activity have been observed, and the heat production of muscle has been more closely analyzed and correlated with the chemical changes. Advances have been made in elucidating the chemical phenomena concerned with the tonic function of skeletal muscle. Excellent experimental work on frog's heart has demonstrated the linkage of the first chemical changes of contraction with oxidative processes.

No essential progress seems to have been made in the analysis of muscle tissue. Improvements were, however, introduced in the methods of lactic-acid determination (67, 86, 95), in the preparation of adenosinetriphosphoric acid (88), of adenylic acid (116), and of inosinic acid (43, 116), in the isolation of acetylcholine (17), and in the determination of hexosemonophosphoric ester (34). The literature of muscle chemistry is still full of papers in which inadequate methods are used. Methods of a more preparative character, with chemical identification of the substances estimated, would surely be desirable in support of results, especially of those obtained by analy-

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¹ The contents of this review will be limited to the periodicals received to December 1932 but a number of papers published in 1931, not considered in last year's treatment of the subject, will be included. The space allowed for this review makes it impossible to refer in the text to every author and every paper.

² *Ann. Rev. Biochem.*, 1, 431 (1932).

³ Literature pertaining to the nitrogenous extractives of muscle, creatine and phosphocreatine, ammonia formation, sulphur compounds, and a portion of that on glycogen, being considered elsewhere in this volume, will not receive treatment in the present section. (EDITOR.)

ses of small quantities of tissue. Steudel (133), for instance, found oxalic acid in beef muscle, in amounts of not less than 14 mg. per cent; it is therefore pertinent to ask, in view of the similarity of oxalic and succinic acid, how often oxalic acid, thus far overlooked, may have been mistaken for succinic acid.

The physical properties of skeletal muscle.—Living muscle obviously has a positive volume coefficient of thermal expansion, but its linear coefficient in the direction of the fibres is negative under small load (147). To this fact may be attributed the thermal response of the living muscle, characterized by a development of heat when the frog sartorius is stretched under a small load (50). Under a heavier load living muscle (and always dead muscle) has a positive linear coefficient of thermal expansion, and its thermal response to stretching is cooling. The thermoelastic properties under small load are the properties of the muscle fibres, while those of the heavily loaded or dead ones are properties of the connective tissue in the muscle.

Mechanical conditions and metabolism.—The intensity of the well-known metabolic changes in resting muscles is increased by increase of tension: *Feng effect* (49).

The volume contraction of the twitching muscle (Ernst-Meyerhof effect).—Contrary to what was a familiar experiment in physiological teaching, the volume of the muscle is decreased during the twitch, synchronous with the mechanical response of the stimulated muscle. Apparently the effect is chemical in nature, and may be considered as a phenomenon of electrostriction. It is interesting that the reactions which are now supposed to follow the contraction, viz., splitting-off of phosphoric acid from adenosinetriphosphoric acid and phosphocreatine, and deamination of adenosinetriphosphoric acid, produce *in vitro* a volume-contraction of their solutions, while lactic-acid production from glycogen or hexosephosphate causes expansion of volume (96).

The decrease of the double refraction of the birefringent disks during contraction (von Murralt effect).—This effect is also synchronous with the mechanical response, and has two maxima, the first corresponding to the development of tension, the second to relaxation. The optical changes are correlated by the author with internal changes in the string-like molecules of myosin (106).

The heat production of the stimulated muscle.—The heat production under anaërobic conditions has been thoroughly analyzed (26,

68, 69). The heat production occurring after the contraction is over—delayed anaërobic heat—is regarded as the balance between the endothermic resynthesis of phosphocreatine and the exothermic formation of the lactates; while the aerobic heat production is the balance between the exothermic oxidation of lactic acid and the endothermic resynthesis of glycogen and completion of phosphocreatine resynthesis.

At low temperatures a phase of cooling may follow the initial heat production. The endothermic restoration of phosphocreatine is driven by the free energy of the chemical change next in order and, at a certain point, the total energy of the simultaneous reactions may be negative (Hartree, 69).

Distribution of water and mineral salts in the muscle tissue.—Interesting conclusions concerning the localisation and function of water in muscle are to be found in Hürthle's papers (74). Concerning the distribution of free water and closely bound water of imbibition, see Ernst (46) and Buccardi (19). The colloid-osmotic pressure of the muscle is treated by Duff (41). The phenomena of subcooling and of ice formation in muscle fibres, studied by microdissection, are discussed in the paper by Chambers & Hale (27). The range of variation of the potassium content in muscles is treated by Bernard (16) and Steffen (132). Leulier *et al.* (84) have reported on potassium in relation to chronaxy in normal and degenerated muscle. The perfusion experiments of Mond & Netter (103) lead to the remarkable conclusion that sodium exists in the muscle in excess of chlorine and that this sodium, in variable amounts, must be concentrated on the external surfaces of the muscle fibres and may be returned to or taken from the perfusing solution. Interesting relations between the fixation of sodium on these surfaces and the recovery changes of lactic acid are suggested.* The permeability of the muscle and its changes in activity have been the subject of numerous investigations, without uniform agreement, however, as to whether there is a difference in the action of direct and indirect stimulation upon changes of permeability for potassium and phosphate ions. For the influence of lactic acid upon the diffusion of potassium and phosphates from perfused muscle, and conclusions as to the rôle of these ions in the mechanism of muscular contraction, see Ernst & Takács (47) and

* Concerning sodium in muscle see Wu (149) and Randois & Michaux (125).

Ernst & Fricker (48). The influence of lactic-acid concentration and of pH upon the partition of lactate between tissue and perfusing solution has been studied by Rowinski (128).

The CO₂-dissociation curve has been established for living dog muscle by Irving, Foster & Ferguson (75), on lines established previously for the dissociation curve in blood. The CO₂-combining-power curve as well as figures for the calculation of the buffering power of muscle are given. The latter is shown to be a function of the carbonic acid-carbonate system, of other weak acids and their salts, and dependent upon the respiratory escape of CO₂. An investigation of the neutrality changes in isolated amphibian muscle was made by Meyerhof, Möhle & Schulz (99).⁵ In these experiments the tension developed and the reaction changes were measured at the same time; phosphates and proteins were taken into consideration as factors determining the pH at given CO₂ concentrations.

The proteins of the muscle.—The criticism directed by Yanagi (150) against the principles of classification of muscle proteins will hardly be accepted after the attainments referred to in the previous review (pp. 433-444). Alterations in the condition of muscle proteins by fatigue and postmortem changes have been observed by Deuticke (37) and Ørskov (120). In the work of Ørskov the swelling of muscle, pulverized while frozen in liquid air, was measured by the volume attained in solutions of H₂SO₄ under standard conditions at 0°. In fatigued muscle the swelling was increased; thawing before stopping the postmortem changes with H₂SO₄ made the volume decrease. These effects were not produced by addition of lactic acid or phosphoric acid, and probably were not due to the presence of these acids; they can be regarded as the effect of changes in the muscle proteins produced by fatigue and postmortem disaggregation. In Deuticke's experiments the decrease of proteins soluble in a standard salt solution (potassium phosphate *M*/11.67, potassium iodide *M*/35, pH 7.2) is stated to be fairly proportional to the "tension \times length" product developed in 30 to 900 twitches. The decrease of solubility is reversible; it disappears when the fatigued muscles are kept in oxygen, but does not disappear in nitrogen. It is remarkable that the changes in the muscle proteins were observed in Deuticke's experiments after extraction of the minced tissue with a neutral salt

⁵ Cf. *Ann. Rev. Biochem.*, 1, 444 (1932).

solution, when all the familiar metabolic changes (viz., lactic-acid formation, deamination, splitting-off of phosphorus from phosphocreatine and adenosinetriphosphoric acid) are known to occur at a very fast rate in the traumatic and postmortem decompositions, and no agent was used to stop them. Possibly essential changes produced by fatigue in the contractile apparatus are observed here; the question as to whether these changes are those of the contraction itself must, however, be left open. The denaturation of muscle proteins by freezing is treated by Finn (55) and the changes in juices from heart muscle by Wassermeyer (145).

Muscle hemoglobin or myoglobin.—This pigment has been isolated in crystalline form by Theorell (140) from the heart muscle. The iron content is the same as in hemoglobin; the molecular weight as measured by the ultracentrifuge method is 35,000, which is half that of blood hemoglobin. The isoelectric point is at pH 6.99—more alkaline than that of hemoglobin—the spectroscopic “span” for the CO compound smaller, and the transformation into methemoglobin easier.

Acetylcholine.—The method previously applied by Kapfhammer and his colleagues (17) for the isolation of acetylcholine from cow's blood was extended by these authors to the analysis of muscle and other tissues; muscle was found to be richest in acetylcholine; 194 mg. per kg. of tissue were found in skeletal muscle and 32 mg. in the heart. Cow's uterus, pregnant or void, did not contain acetylcholine, but the highest concentration of choline, of all organs investigated, was present in the uterus. As the occurrence of acetylcholine in blood has been a matter of controversy, the present reviewer wishes to emphasize the fact that he has been able to confirm Kapfhammer's findings, and that he therefore accepts the statement of Kapfhammer without reservation. The isolation of acetylcholine from skeletal muscle and heart muscle beautifully substantiates many previous assumptions. Acetylcholine has long been considered as the “*Vagus-stoff*,” the substance which is liberated by the stimulated vagus in the effector organs and which produces the nerve's effects. It is supposed to be the chemical factor whose presence is responsible for producing and maintaining the tonic contraction⁶ of skeletal muscle,

⁶ For the tonic function of muscle see Bremer's comprehensive article (18), Freund's short review (56), and Riesser's paper (126), also the contributions of Wachholder and his co-workers (82, 83, 115, 141, 142, 143).

in which it was detected by pharmacological assay. The contractures produced by acetylcholine are, of all chemical contractures, the only ones which can be likened to physiological contractures or tonic contractions. The clasping reflex of the male frog can be reproduced by the action of acetylcholine; the low intensity of metabolic changes during acetylcholine contracture in isolated muscles [Miura (102); but see also Hegnauer (70)], in which maximal contracture is produced by minute acetylcholine doses, corresponds to what is considered characteristic of tonic contractions. Plattner (122, 123) finds that the sensitivity for acetylcholine in amphibian and turtle muscle varies with the amount of acetylcholine present in the muscle and its fitness for tonic function; parallel seasonal variations are also noted. For a discussion of the special character of acetylcholine as a contracture-producing agent and the relation between susceptibility to this agent and the seasonal variations in tonic function, see Gellhorn & Northup (58).

The purine derivatives.—The great interest in the nucleotides and their polyphosphoric derivatives is accompanied by great improvements in methods of preparation. Both adenylic acid, prepared from butcher meat or from yeast, and inosinic acid are now fairly cheap commercial products. The structure of adenosine is established by Levene & Tipson (85); adenylic acid and its deamination product possess the structure of 7-adenine-furanoriboside-5-phosphoric acid. The preparation of adenosinetriphosphoric acid⁷ has been improved by Lohmann (88), while modified methods of preparation and new salts are reported by Barrenscheen and his colleagues (10). The preparation of inosinetriphosphoric acid, both by enzyme and nitrous-acid deamination of the adenosinetriphosphoric acid, has been described (88, 97, 98, 100). The structure of adenosinetriphosphoric acid is, however, subject to disagreement. The claims of Barrenscheen and his colleagues can be summarized as follows: (a) Adenosinetriphosphoric acid prepared by them from rabbit muscle gives a silver salt with 3 atoms of silver; (b) the acid prepared from this salt does not undergo deamination, either by action of nitrous acid or by the action of muscle-deaminase extracts; (c) by the action of nitrous acid on the original adenosinetriphosphoric acid ino-

⁷ This designation will be used instead of the name pyronucleotide, which was employed by the present writer in the preceding volume of the *Review*.

sinic acid is obtained, along with a modified adenosinetriphosphoric acid which gives a silver salt with 4 atoms of silver; (*d*) the enzymatic deamination of adenosinetriphosphoric acid occurs only after the previous splitting-off of two phosphate groups by pyrophosphatase (prepared by Jacobsen from liver); (*e*) the adenosinetriphosphoric acid which is prepared from the salt with 4 atoms of silver acts as the true coenzyme for the formation of lactic acid in muscle. Barrenscheen and his co-workers conclude that the first adenosinetriphosphoric acid contains the diphosphoric group, on the one side bound to the amino group of adenine, and on the other side esterified with the hydroxyl group on the third carbon of ribose. The second acid, prepared by the action of nitrous acid, has the diphosphoric group linked only to the amino group of adenine. The consequences of this would be very important because, if the structure of adenosinetriphosphoric acid were the one proposed by Barrenscheen, deamination in muscle could proceed only by the simultaneous splitting-off of two phosphoric acid groups or after dephosphorylation, but never before.

The claims of Barrenscheen are opposed by Lohmann (88). The chemical and the enzymatic deamination of adenosinetriphosphoric acid and of adenylic acid proceed at the same rate, and both processes lead to the formation of inosinetriphosphoric acid, the existence of which is not compatible with Barrenscheen's formula. In adenosinetriphosphoric acid the hydroxyl groups on carbon atoms 2 and 3 cannot be esterified since the substance gives the Klimek-Parnas reaction (80) for adjacent hydroxyl groups (88). Barrenscheen has not yet published a reply, but the present reviewer considers the direct evidence brought forward by Lohmann as conclusive.

There are indications of the existence in fish muscle and erythrocytes of adenosinepolyphosphoric acids different from the one occurring in muscle [Meyerhof & Lohmann (97)]. A nucleotide, different from adenylic acid, and supposed to be cytosinenucleotide, is found in cow's heart [Drury (40)]. An adenosinepolyphosphoric acid distinct from the one found in muscle has been mentioned by Lohmann, who found it in heart muscle. Embden also refers to such a substance (*Herznucleotid*), which is supposed to contain phosphorus and nitrogen in the proportion 5 : 10, and is supposed to be a dinucleotide of adenine. Chemical particulars are not given; the physiological action seems to be the same as that of adenosinetriphosphoric acid [Deuticke (39)]. The cozymase of alco-

holic fermentation, also an adenine-phosphorus compound, but different from adenylic acid and its polyphosphoric derivatives, is found in small amounts in vertebrate muscle—more in rabbit's than in frog's; it undergoes synthesis to a polyphosphoric acid, which acts as coenzyme in lactic-acid fermentation of muscle extracts (97).

Kerr concludes that cleavage of nucleotides into free purine derivatives (79) takes place in exhausted, isolated muscles. On the average (of 8 experiments with 60 frogs) the fresh muscles contained 2 mg. per cent of non-nucleotide purine nitrogen and the exhausted muscles 4 mg. per cent. The increase is important when related to the basal value but is small in comparison with the total purine-nitrogen content.

The hexosephosphoric esters and their transformation.—The method of estimating Embden's hexosemonophosphoric ester devised by Cori & Cori (34) consists in the isolation of the barium salt of this ester, and the subsequent determination of the hexose (by reduction value) and the phosphorus (after ashing). The figures for phosphorus and for reducing sugar agree very well in fresh muscle, but when inosinic acid is present the determination of reducing sugar is the more reliable.* For rat muscle, excised during amytal anaesthesia, the following figures are given for the phosphorus distribution in the trichloroacetic filtrate: out of 190 mg. per cent of total phosphorus, 110 mg. belong to inorganic and phosphocreatine phosphorus, 64 mg. to adenosinetriphosphoric acid, 9 mg. (corresponding to about 50 mg. per cent hexose) to Embden's ester, and 7 mg. remain unidentified. No appreciable amount of adenylic acid or inosinic acid seems to be present in the fresh muscle under these experimental conditions. Killing increases the concentration of hexosemonophosphoric ester to 200 mg. per cent (as hexose); injection of adrenalin also produces an accumulation of this ester, and the same effect follows the administration of insulin, probably because of a secondary output of adrenalin.

Cori & Cori regard the Embden ester as a stabilized product of the transformation of a more labile ester which, in turn, is the intermediary product of the breakdown of glycogen to lactic acid; this labile ester is the precursor of the initial lactic acid, while the Embden

* For a discussion of the method of Embden & Jost, see 34, p. 577, and 81, p. 155.

hexosemonophosphate is considered to be the precursor of the delayed lactic acid. The experimental results of the Coris are confirmed by Bell (15). Their conception of the twofold hexosephosphate precursor of lactic acid has been developed in a somewhat related fashion by Buell and her co-workers (20). G. T. Cori (35) gives a balance sheet for glycogenolysis, including hexosemonophosphate, in muscle incubated anaerobically for two hours; criticism is directed against the paper of Anderson and Macleod.⁹

The work of Embden & Jost on hexosephosphate in isolated amphibian muscle has been extended to living frogs by Kraft (81). Here the increase of hexosemonophosphoric ester is observed at the onset of fatigue with accumulation of lactic acid, but afterward the amount of lactic acid and of Embden's ester is said to decrease. The accumulation and subsequent decrease of these substances are supposed by Kraft to be factors which determine the "dead point" and second wind in exercise. The influence of the following factors on the formation and the breakdown of hexosephosphoric esters in muscle have been studied by routine methods: pH (137); Ca⁺⁺ (136); insulin and adrenalin (108); K⁺ and insulin (103a); arsenates (135); hunger, pancreatectomy, and insulin (145a). The experiments of Buell, Strauss & Andrus (20) are remarkable for the methods employed and for the balances of the carbohydrate and phosphorus changes, but there is space left neither for inosinic acid nor for diphosphoric ester, which probably are formed in the minced muscle.

Alactacid muscular activity.—This seems to be observed not only under artificial conditions *in vitro*, but *in vivo* as well, since evidence is accumulating of impaired lactic-acid formation in pathological lesions of the adrenals. In adrenalectomized rats both muscular weakness and a marked impairment of lactic-acid formation are observed. Muscles of such animals even in postmortem rigidity contain less than one-third of the lactic-acid content of normal rat muscle in rigor [Arvay, Verzar & Lengyel (1, 2)]. Similar evidence is presented by Buell, Strauss & Andrus (20). These observations are important as an explanation of the muscular weakness in Addison's disease, in which the energy for the phosphocreatine resynthesis seems to be either lacking, or furnished only at a reduced rate by the exothermic lactic-acid formation. This connection between the adrenal

⁹ Cf. *Ann. Rev. Biochem.*, 1, 437.

constituents and the processes of glycolysis may be considered in relation to the rôle of hexuronic acid in the processes of cellular metabolism as postulated by Szent-Györgyi for the acid discovered by him in the adrenal cortex.

Important information concerning glycolysis is furnished by the extensive studies on lactic-acid formation in various tissues of Haarmann and his co-workers (62, 105). The experiments were performed on minced muscle under standard anaërobic conditions. The following remarkable conclusions are drawn: while, for the normal skeletal muscle, glycogen is the principal source of lactic acid, glucose is a prominent source in heart muscle. In smooth muscle (human uterus) glucose acts as the sole precursor. In foetal muscle and in muscles degenerated after denervation (in such muscles the tonic function and susceptibility to acetylcholine appear), as in smooth muscle, glucose is the precursor of lactic acid. Similar differences are found [Mundelein (105)] for the pigeon. Here the breast muscles—rapidly contracting skeletal muscle—form lactic acid from glycogen, while in the leg muscles lactic acid is formed from glucose and hexosediphosphoric ester.

Resynthesis of glycogen in recovery.—In decerebrated and eviscerated cats Long & Horsfall (89) find a ready conversion of injected *I*-(+)-lactate¹⁰ into glycogen in the presence of glucose and insulin; less glycogen is formed from lactic acid and glucose alone, and still less from lactic acid alone. For a discussion of the mode of conversion of lactic acid into glycogen and a refutation of too simple explanations see Dean Burk (21). The scheme deduced from the experiments of Hoet and his school¹¹ is rendered somewhat dubious by the findings of Cleghorn & Peterson (33), who report a partial reappearance of glycogen in the muscles of fully eviscerated and decerebrated cats even without insulin infusion. For the lactic-acid cycle see Shorr, Loebel & Richardson (129) and Nitzescu & Benetato (114). The disappearance of lactic acid in perfused rabbit muscle is found by Ørskov (118) to proceed at a rate equal to that observed in experiments on hepatic perfusion, the comparison being

¹⁰ The designation accepted in chemistry will be employed here: *I*-(+)-lactic acid means the dextro-rotatory natural lactic acid occurring in muscles, designated previously as *d*-lactate.

¹¹ Cf. *Ann. Rev. Biochem.*, 1, 436.

based upon equal weights of both tissues. An anaërobic disappearance of lactic acid in minced muscle was observed by Haarmann in experiments in which formation of lactic acid was prevented by bromoacetic acid. The author finds that under the same conditions lactic acid may be produced from pyruvic acid and from substances that can be transformed into pyruvic acid in the muscle pulp (62).

Pyruvic acid and pyruvic aldehyde.—The dismutation of synthetic pyruvic aldehyde or phenylglyoxal by muscle extracts, inactivated by dialysis or oxygen, requires the presence of reduced glutathione [Lohmann (87)]; the transformation of glycogen into lactic acid, however, does not require the presence of glutathione, nor is this agent necessary for the enzymatic Canizzaro dismutation of aldehydes. For the chemical transformations of pyruvic aldehyde, the papers of Neuberg *et al.* (111, 112, 113) should be consulted. Hahn (65) has summarized his work on dehydrogenation reactions in muscle pulp. Pyruvic acid appears as the final product of dehydrogenation of succinic acid, fumaric acid, malic acid, citric acid, of Embden's hexosemonophosphoric ester and Harden-Young's diphosphoric ester, and possibly also of glycerophosphoric acid and propionic acid. These dehydrogenations proceed in the absence of oxygen or of other added hydrogen acceptors; the acceptors normally present or those produced by the muscle pulp from its own constituents are adequate. These findings are extremely important and should be taken into consideration in all theories pertaining to muscle chemistry and muscle metabolism; muscle contains an abundant supply of hydrogen carriers and acceptors which provide the means of hydrogen transport. The formation of lactic acid from pyruvic acid and related substances belongs to this field. No conclusions can be drawn, however, from the list of such changes occurring in muscle pulp, as to the real metabolic changes in the muscle, living or surviving. The experiments of Case (24) differed from those of Hahn and Haarmann: muscle extracts were used and aldehyde was fixed by the addition of sodium bisulphite. In these experiments pyruvic acid did not appear as an oxidation product of lactic acid, but rather as the transformation product of an intermediary between the carbohydrate and lactic acid, probably pyruvic aldehyde. Pyruvic acid was formed from starch, but much less from added lactic acid; inhibition of glycolysis suppresses pyruvic-acid formation, while "antiglyoxalase" seems rather a favourable factor.

Barrenscheen & Braun (12) refer to a large disappearance of acetic aldehyde from muscle pulp, following the addition of adenosinetriphosphoric acid, and of a still greater disappearance after adding Harden-Young hexosediphosphoric ester. Such participation of aldehydes in the breakdown of glucides may contribute to an understanding of the finding, by Martini & Crocetta (93), of alcohol formation in muscle, assuming that the alcohol has been satisfactorily identified.

The amount of the still unidentified, ether-soluble, phosphorus- and nitrogen-free acids, other than lactic acid, is found to be increased in the muscle after activity or asphyxia to 3 times the resting value [Ørskov (119)].

Muscle respiration.—For the oxygen consumption of muscles rendered inexcitable, see Fenn (51), and for the striking phenomenon of CO-combustion by muscle and heart, see Fenn & Cobb (53).

Banga, Schneider, Szent-Györgyi & Vargha (5, 6, 7, 8, 9) claim to have isolated the respiratory system of pig's heart muscle in the following way. From the boiled, trichloroacetic-acid muscle extract one fraction was obtained, which contained adenylic acid and hexosephosphoric acid; this fraction in combination with the washed muscle pulp consumed oxygen; this respiration was not affected by the presence of arsenates. The other fraction, containing a yellow pigment and giving sulphur and pentose reactions, formed with the washed muscle-pulp a system which consumed oxygen and oxidized lactic acid to CO_2 and water. This oxidation did not require the presence of phosphates and was stopped by the addition of arsenates.

The respiration of muscles poisoned with bromoacetic acid is found to be 60 to 70 per cent of the normal resting consumption of O_2 ; the excess consumption after stimulation does not seem to produce a functional recovery [Wright (148)]. For the oxidation of lactic acid in muscles poisoned with iodoacetic acid, see Mawson (94). The metabolism of frog muscle after reduction of the carbohydrate reserves by insulin was studied by Gemmill (59); the respiratory quotient of such muscles is only slightly below the normal, but in a number of experiments with muscles largely deprived of glycogen the lactic-acid production is much smaller than expected from the tension \times length product. The respiratory quotient of normal resting frog muscle is found by Fenn to be lower than the usually quoted value. In summer the average is 0.85, in winter 0.87 (52).

The sequence and linkage of chemical changes in the muscle.—The knowledge of this field has changed but little in the past year. Much evidence concerning the splitting and resynthesis of phosphocreatine, adenosinetriphosphoric acid, and lactic-acid formation from the point of view of energetics and linkage of processes has been published by Meyerhof & Lohmann (98).

In muscle extracts phosphocreatine is synthesized, when the reaction is alkaline and an adequate energy source is available; for this endothermic synthesis 12 large calories per mol are necessary, and these are supplied by the breakdown of adenosinetriphosphoric acid, or by lactic acid. In the breakdown of adenosinetriphosphoric acid, 25 large calories are liberated when two phosphate groups are split off and 8 when the substance is deaminized. A resynthesis of adenosinetriphosphoric acid from adenylic acid also occurs in muscle extracts, but only at the expense of energy from glycolysis which is activated by the presence of adenosinetriphosphoric acid. Inosinetriphosphoric acid may replace adenosinetriphosphoric acid in the coenzyme system but inosinic acid cannot be substituted for adenylic acid. Induction of esterification in inactivated muscle extracts by both pyronucleotides has been observed by Milroy (100).

This striking linkage of exothermic cleavages with endothermic syntheses in structureless muscle extract makes similar linkages in muscle the more probable; it must be mentioned, however, that we have now to consider an increased number of substrates and enzymes of muscle tissue, and it is therefore rendered more difficult to select the real path of metabolic change.

The two possible paths of decomposition of adenosinetriphosphoric acid have been studied by Mozolowski, Reis & Sobczuk (104), and by Lohmann (88). The formula of Barrenscheen & Filz (13), if correct, would exclude the possibility that deamination is the first process. In Lohmann's experiments, however, traumatic deamination of short duration, at room temperature, leads to inosinetriphosphoric acid; deamination consequently may precede dephosphorylation. In the experiments of Mozolowski deamination can be suppressed at an alkaline reaction, while dephosphorylation goes on. In unpublished experiments of Mozolowski, Sobczuk & Baranowski the two processes are shown to have different temperature coefficients; when frozen muscle powder is thawed and brought to definite temperatures for limited periods of time, deamination comes to the fore as the temperature is elevated.

Very remarkable information concerning the linkage of the resynthesis of phosphocreatine either with glycolysis or with oxidations is derived from the experiments of Clark *et al.* (29, 30, 31, 32) on the isolated perfused frog heart. Previous experiments by Clark, Gaddie & Stewart have demonstrated that such hearts, when the oxygen supply is good, bring about a marked decomposition of amino acids, and that they are not at all favourably affected by the presence of glucose in the perfusing salt solution. Now, when perfused with Ringer solution containing too few calcium ions, the heart is found to contain more carbohydrate than the control; the source of this gluconeogenesis is unknown, but can be postulated to be the material deaminized in the aerobic heart. Under anaërobic conditions the heart uses up carbohydrate when perfused with a well-buffered alkaline Ringer solution and produces lactic acid: glycolysis is the source of energy when the oxygen supply is deficient. The carbohydrate breakdown in the heart is stimulated by anaërobiosis.

In anaërobiosis the phosphocreatine in the turtle auricle or the frog ventricle is decreased, and the phosphagen coefficient (phosphocreatine P; total P) falls from about 1 to about 0.1, and remains for a long time at this level: a steady state of phosphocreatine breakdown and resynthesis is attained. When the frog heart is poisoned by iodoacetic acid, the beat may remain unchanged in oxygen, but stops very soon when oxygen is removed from the perfusing liquid. We have here a demonstration of the linkage between the successive processes in muscle restitution presented most lucidly, in experiments which cannot be performed on skeletal muscle with the same degree of reproducibility. The breakdown of phosphocreatine is the primary change, occurring with or after the contraction. The resynthesis of phosphocreatine may go on either at the expense of glycolysis, when the oxygen supply is deficient, or at the expense of energy supplied by oxidative changes; it stops and the heart beat ceases when neither oxygen is present nor glycolysis is possible.

The opinion frequently pronounced by those who in the last few years have reviewed the chemistry of muscle can be summed up in the statement that we know much more in this field than six years ago, when our ideas were too simple indeed, and that we are now much better aware of the limits of this knowledge. The time is not yet ripe for the development of detailed, concrete ideas as to the nature of muscular contraction, relaxation, and recovery. The general observations on theories of muscular contraction made by Ritchie (127)

are remarkable, however, for an entirely new point of view, and because they admirably fit both the old and the new knowledge of muscle chemistry. The view has been held that the energy for muscular contraction is supplied by the chemical processes stated to occur when the muscle contracts and supposed to be previous to or simultaneous with the contraction itself. This view was accepted since Meyerhof had proved that the formation of lactic acid and the other processes released by the lactic-acid formation were sufficient to account for the energy changes in the muscle cycle. Now, the lactic-acid formation is regarded as a more distant step in the recovery processes, and other changes, recognized to be nearer to the contraction, are supposed to release the energy for contraction.

Ritchie views the problem in a different way. He considers the contractile mechanism, whatever may be its nature, to be charged at rest to a steady high potential, which is maintained by the resting metabolism. On excitation, the potential falls to zero and the muscle tends to shorten: the shorter length corresponds to the discharged state. It then relaxes because the contractile mechanism is recharged to its original high potential by what is essentially a speeding-up of the chemical changes occurring also in rest. In quick muscles the recharging is begun by the rapid process of phosphocreatine breakdown, followed by other recovery changes. In this remarkable theory a function is provided for the resting metabolism, an explanation of the qualitative identity of the resting metabolism and the metabolism of activity. The refractory period after excitation is accounted for. Even the distinction that some muscles may be self-exciting and others not can be deduced. The initial resting state may be the more unstable the higher the potential; in heart muscle the recharging is supposed to be slow, but it tends to charge up to the limit of stability, and an automatic discharge may follow. In skeletal muscle the charging is rapid, but does not exceed the limit of stability, and the extra energy supplied by the stimulus discharges the system. From this point of view all the chemical reactions we know in the muscle appear as reactions of relaxation and recovery. The understanding of the essential reversible mechanism is still beyond our knowledge.

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DEPARTMENT OF MEDICAL CHEMISTRY
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CHEMICAL EMBRYOLOGY*

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THE EGG AS A PHYSICO-CHEMICAL SYSTEM

The permeability of the membranes surrounding eggs continues to attract a great deal of attention. M. Smith has extended his work on the osmotic relationships in the hen's egg (summary in Needham, Smith, Shepherd, Stephenson & Needham) by studying the behaviour of yolks immersed in solutions of electrolytes and non-electrolytes, and by further dialysis experiments. Bateman, vindicating the reliability of the Hill vapour-pressure method, has again demonstrated the existence of a real osmotic difference between avian yolk and white, and draws attention to the removal of osmotically active substances which occurs when yolk and white are mixed. Howard (1), on the other hand, claims, as the result of freezing-point determinations, dialyses, and vapour-pressure measurements, that the yolk and white have the same osmotic pressure. Her conclusions are not accepted by Smith & Shepherd, and the subject cannot as yet be considered settled. Meanwhile Gray has given a discussion of the comparative differences between the membranes of hen's egg and trout egg, in which he concludes that the vitelline membrane of the latter possesses static impermeability to water and electrolytes. Similar conclusions for other teleost eggs are indicated by Adler's work with dyes.

The shell-membranes of the hen's egg have also received attention as regards osmotic properties, potential differences, et cetera, by Osborne; Mizutani; Yasumaru; Yasumaru & Sugiyama, and Szuman. More important biologically are the numerous recent researches on the permeability of the cell-membrane of the echinoderm egg. Anomalous swelling of sea-urchin eggs is reported by Dorfman. The effect of various factors on the permeability of these cells is also considered—formaldehyde, Hykes; ageing, Goldfarb & Schechter; maturation, Thörnblöm; activation, R. S. Lillie and Favilli. Other papers which have appeared on this subject are those of Jacobs & Stewart; Stewart & Jacobs; McCutcheon, Lucké & Hartline; McCutcheon & Lucké; Haywood & Root; Lucké; and A. R. Moore, while much other literature has been discussed in the valuable review

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of Lucké & McCutcheon. The potential difference across the nuclear membrane of the starfish egg has been investigated by Gelfan; the nucleus is from 4 to 21 millivolts positive to the cytoplasm, and there is no potential difference between cytoplasm and sea-water.

A good deal of work has been done on the surface tension and viscosity of invertebrate eggs, especially with the new microscope-centrifuge, by Harvey (1); Cole; Cole & Michaelis, and Howard (2). The last-named worker, considering the viscosity of the echinoderm egg under various shearing forces, concludes that a continuous semi-rigid structure is not a fundamental attribute of egg-protoplasm, but that it is, on the contrary, a true liquid [cf. the discussion in Needham (7)].

Turning to the chemical constitution of the egg, we may first refer to the work on the hen's egg, which, as usual, exceeds in amount the rest of the literature. The changes which occur during development in the various parts of the egg in pH and water-content have been followed anew by Rubinstein and by Orru and Saccardi & Latini respectively, but without the addition of many new facts. As regards the egg-white, Svedberg finds that fresh ovalbumin has a much lower sedimentation-constant on the ultra-centrifuge than the purified protein, and concludes that the former exists *in ovo* in a form widely different from the latter (molecular weight 34,500). Accurate studies of the amino-acids of crystalline ovalbumin are reported by Vickery & Shore and by Calvery (1), and the specific rotation of the egg-white proteins, by Holst & Almquist. The effect of diet on the composition of the proteins is under investigation by Calvery & Titus.

Romanov (1) has studied the chemical growth of the avian ovum (calcium, fat, water, pH, etc.) by ordinary estimation methods, and Marza has made a parallel study by means of histochemical technique. The amino-acids of vitellin have been studied by Calvery & White. Earlier views on the constitution of vitellin are confirmed by Lipmann & Levene, who report that all its phosphorus exists in the molecule in the form of serine phosphoric acid. The subject of the organic iron in the yolk has been reopened by McFarlane, who believes that lecitho-vitellin contains iron in organic combination, but not so strongly bound as to be inactive toward Hill's dipyridyl reagent [see Needham (1), pp. 1368 ff.]. The further rehabilitation of "haematogen" will be awaited with interest. Meanwhile McFarlane's results are to some extent supported by the histochemical findings of Marza, Marza & Chiosa. McFarlane also brings forward

evidence for the existence of organic copper in the yolk, and describes experiments on the alkaline hydrolysis of lecitho-vitellin. The basic amino-acids of livetin (the second protein of yolk) have been examined by Jukes & Kay (3), and the protein itself has been shown by the same workers (2) to be immunologically indistinguishable from avian serum globulin. They have also given us an excellent review of yolk-protein chemistry [Jukes & Kay (1)]. Other constituents of the hen's egg which have been investigated are cholesterol (Gaujoux & Krijanovski), lecithin (Lintzel; Bornmann), ash (Alfend), phosphorus (Fitelson & Gaines), iodine (Jaschik & Kieselbach; Scharrer & Schropp), manganese (Peterson & Skinner). Shell texture has been studied by Holst, Almquist & Lorenz, shell fluorescence by Bierry & Gouzon, and shell formation by Asmundson. The now discredited view that shell production is associated with thymus function is discussed (with bibliography) by Greenwood & Blyth and by Riddle & Krizinecki.

The eggs of the lower vertebrates have been dealt with in two magnificent ecological and physiological reviews by Wunder on fishes (1) and amphibia (2), which will be of great service for chemical investigators. Another valuable compilation is that of Harvey (2), who has collected together all the physical and chemical constants relating to echinoderm eggs. Of the detailed contributions, the most interesting is probably that of May, who finds that in the eggs of the snail, *Helix pomatia*, glycogen is completely replaced by galactogen (sinistrin), which accounts for as much as 37 per cent of the dry organic substance. In the adult snail, glycogen and galactogen occur together. Is galactogen characteristic of all mollusks?

Inclusive analyses of eggs have been made by Schmidt-Nielsen & Stene (for the skate *Raja oxyrrhynchus*; very low in fat) and by D. E. Smith and Miller & Smith (for the sea-urchin *Echinometra lucunter*). Glutathione has been estimated in silkworm eggs by Kitamura, and the fatty acids of orthopteran and other eggs studied by Slifer and by Terroine, Hatterer & Roehrig. Chambers has reinvestigated the pH of *Fundulus* egg-protoplasm, and Mayer has discovered a remarkable transition vaterite → aragonite → calcite in the calcium carbonate of the developing gastropod embryo. Ultra-violet light is stated by Sypniewski to dissolve away amphibian egg-jelly without injury to the eggs, and Bank has made a report on the jelly of the sea-urchin egg. Finally, an important paper on the biometrics of insect eggs is due to Alpatov & Bachvalova.

GROWTH AND DIFFERENTIATION

Important theoretical papers in this field have been those of Shumway on the recapitulation theory and Haldane on the time of action of genes in embryonic development. We are given a classification of genes according to the time at which they act in ontogeny, which may be distributed over more than one life-cycle. The importance of this, both for biochemistry and evolution-theory, is considerable. Next, the dissociability of the fundamental processes of growth, differentiation, and metabolism has been the subject of a review by Needham (7), which organises the known facts on the basis of an analogy with mechanical gearing. The Huxleyan treatment of relative morphological growth (recently made available in monographic form) has been extended by Needham (3, 4) to relative chemical growth. The heterogeneity of the principal chemical constituents of the chick embryo has thus been ascertained, and in many cases turns out to be identical with that of the same constituents in other developing organisms, revealing, it is suggested, the chemical ground-plan of metazoan growth.

EMBRYONIC RESPIRATION

The rise of respiratory rate following fertilisation [cf. Needham (1), Sec. 4.2], which has often been regarded as of general validity, now fails to appear as new organisms are examined. The O₂-consumption of *Cumingia* (mollusk) and *Chaetopterus* (annelid) eggs decreases 50 per cent [Whitaker (1)], that of *Nereis* (annelid) eggs increases [Whitaker (2)] or remains the same (Barron), that of *Asterias* (starfish) eggs remains constant [Tang (1)]. Rhythmic variations following mitosis have been found to be absent in echinoderm development by Tang (2), but are reported for the frog's egg by Brachet (3). The oxygen-tension/oxygen-consumption curve for sea-urchin eggs is given by Tang & Gerard.

Perhaps the most important work this year on embryonic respiration is that of Runnström, who has studied the increase of respiration in sea-urchin eggs to which paraphenylenediamine has been added, and in which it acts as a non-autoxidisable oxygen-transportase. Inhibition experiments with KCN and CO prove that in the unfertilised egg the "Atmungsferment" is fully active, but "unsaturated" (Örström). Similarly, Friedheim obtains 15-fold increases of respiration when echinoderm eggs are treated with echinochrome or a pigment derived from polychaete worms.

For the chick embryo an extensive study of respiratory metabolism by Needham (5) has been proceeding. The R.Q. of blastoderms at different stages has been measured and also that of the yolk-sac and allantois throughout development. The effect of fluoride, iodoacetate, et cetera, on the respiration and R.Q. of embryo and membranes has been studied, and it has been shown that the former, when in the "carbohydrate period," can combust protein if glycolysis is fully inhibited. Normal differentiation *in vitro* (Waddington) proceeded in concentrations of KCN sufficient to reduce the respiration to minimal proportions. Other work is that of Kiyohara on the respiration of chick retina, Lipmann & Fischer, and Lipmann on that of chick explants, Duryee on *Ambystoma* embryo respiration at different osmotic pressures, Kisch on the effect of aluminium, borate, et cetera, on mammalian embryo tissue respiration, Cunningham & Reid on the secretion of oxygen for the eggs by the pelvic filaments of the lung-fish (*Lepidosiren*), Alt & Tischer on the R.Q. of tapeworm eggs. Needham (6) has observed a diminution of R.Q. during the development of crab eggs, a fact which is significant for the succession of energy-sources in ontogenesis [see Needham (1), Sec. 7.7].

The decline of metabolic rate with age has been extensively studied by Brody and his colleagues, and by Riddle, Nussmann & Benedict, for mammals and birds. It has also been extended to mollusk tissue (Hopkins) and daphnids (Terao).

Outstanding among mammalian work is that of Haselhorst & Stromberger (2) on the gas-tensions of human umbilical and maternal blood, and the gas exchange in the placenta, which they find to be purely a process of diffusion (see also de Candia). Hoag & Kiser give data for the acid-base equilibrium of foetal blood, and Reiss has discovered a remarkable resistance to asphyxia on the part of newborn rats. For the basal metabolism in pregnancy see Schwarz & Drabkin; for foetal heart rate see Hartman, Squier & Tinklepaugh (monkey) and Bogue (chick); for foetal blood pressure see Clark (cat); and for the blood volume of the pregnant uterus see Barcroft & Rothschild (rabbit).

CARBOHYDRATE METABOLISM

The most original contribution here is that of Dickens & Greville, who have studied the fructolysis of embryonic tissues [cf. Needham (1), Sec. 8.14]. Wide variations in intensity of the attack on

fructose are found, and it is thought that they may be accounted for by variations in ease or extent of phosphorylation. Another interesting line of work is that of Kataoka, who has injected various carbohydrates into the hen's egg early in development; in this way glucose, fructose, mannose, galactose, maltose, and lactose were found to be precursors of glycogen. Pentoses, glucosamine, and oxymethylfurfural were not. According to Lund, the glycogen of embryonic tissues differs from glycogen prepared from adult liver. Using the explantation technique, Watchorn & Holmes have tested on embryo-rat kidney the protein-sparing action of fructose, galactose, and xylose; all these are utilised by the cells, but only the first-named inhibits the production of ammonia and urea. Pentimalli claims that embryo extract inhibits the anaerobic glycolysis of muscle.

On general carbohydrate metabolism, especially glycogen, there are papers by Tirelli (for the silkworm egg), Biasiotti (chick), Dydovska (*Ascaris*), Kurkiewicz (echinoderm), Nakamura (mammalian foetal liver), and Demuth and Krontovski (explanted mammalian embryo cells). Placental glycogen and the factors affecting it are dealt with by Rabinovitch, by Murakami (1 and 2), and by Rabe; the latter shows that the glycogen of placenta and foetal liver is unaffected by alimentary hyperglycaemia in the maternal organism [cf. Needham (1), pp. 1018 ff.].

NITROGEN METABOLISM

A solid piece of work on this subject has been done by Schenck, who traces the distribution of the proteins and the changes in composition which they undergo in yolk, white, membranes, embryo, et cetera, of the chick. Terminology and technique make the results a little difficult to interpret, and the same may be said of the other large recent research in this field, namely that of Wilkerson & Gortner on the nitrogen metabolism of the developing pig embryo. The Van Slyke methods for protein analysis were here applied to whole embryo tissue after acetone-alcohol-ether extraction, so that the results, although comparable one with another, are hard to relate to anything else in the literature, except the earlier work of Gortner himself on aquatic eggs [see Needham (1), pp. 1108 and 1111]. The paper, however, includes valuable data for water, sulphur, glutathione, ash, et cetera. More satisfying is the less ambitious study of Calvery (2) on the amino-acids of totally hydrolysed yolk, white, embryo, and

shell membrane throughout development. In percentage, tyrosine decreased in the embryo but remained constant elsewhere; tryptophane and cystine increased in the embryo, remaining constant elsewhere; arginine and lysine were constant throughout; histidine everywhere decreased. According to Ido, there is no change in the proline and hydroxyproline content of hydrolysed whole eggs during development. Impallomeni finds asparaginase in chick embryos, and Goldberger (surely mistakenly) obtains no free amino-acid nitrogen from seventh-day chick embryos. This has bearing on the growth-promoting proteose, which, according to Borger & Zenker, can suffer drying of embryo extract containing it without injury. Further on this subject see Willmer & Kendal, who find the growth-promoting action of pure proteoses only to be exhibited on explants in embryo extract or plasma, not in saline. Deamination by embryo tissue *in vitro* has been studied by Glover, who finds a slight action.

The contradictions regarding the nuclein metabolism of echinoderm eggs [see Needham (1), p. 1158] have now been settled by Brachet (1, 2) who finds augmentation of thymonucleic acid (Dische method) and decrease of "pentoses" (Hoffmann method) during development. It is sure, then, that a *d*-ribose or "phyto"-nucleic acid passes into a *d*-ribo-desose or "thymo"-nucleic acid, thus satisfying the earlier determinations of constancy in nuclein phosphorus and purine nitrogen. Silberberg & Voit and Beck & Truszkovski have also been occupied with purine metabolism in embryonic life.

On the protein content and osmotic pressure of mammalian foetal and neonatal blood, see Clark & Holling. Changes in the vitellin molecule during development of turtle eggs are reported by Kusui (2).

FAT AND LIPOID METABOLISM

A straightforward study of the fat utilisation by the chick embryo (estimations in embryo and yolk) has been carried out by Romanov (2), and this is paralleled by the histochemical work of Konopacka, and the physiological work of Remotti on yolk-absorption. The properties of the fat of the hen's egg have been followed during development by Kusui (1). Free fatty acids reach a maximum on the third day of incubation; the number of double bonds in percentage of the carboxyl groups remains roughly constant. Embryonic cholesterol metabolism has been dealt with by Cattaneo (2) and by Roffo. As regards cerebrosides, an important paper on myelinogen-

sis and initiation of movement has appeared by East, who considers that myelinogenesis cannot here be the limiting factor. Keene & Hewer have written on "bursts" of myelinisation in mammalian embryos.

Much attention has been devoted to "pigeon's milk," a subject of importance for foetal nutrition, since the squab is hatched in an undeveloped state. The "milk" is a white slimy fatty material, formed in the crop of the parent birds by desquamation of epithelial cells, i.e., a sacrifice of cell-structure for the nutrition of the young. Beams & Meyer have given a general account of it; Reed, Mendel, Vickery & Carlisle and also Dabrovska have studied its chemical and nutritional properties; Kaufman & Dabrovska; Riddle & Dykshorn, and Riddle, Bates & Dykshorn have investigated its endocrine control mechanisms.

For the eggs of certain worms and mollusks, histochemical data have been made available by Konopacki. For mammalian livers there is work by Roussel & Deflandre and for other tissues by Cattaneo (4).

INORGANIC METABOLISM

By micro-incineration methods Horning & Scott have attempted to chart the composition and amount of ash in sections of chick embryos early in development, and in explanted cells (Scott; Scott & Horning). For mammals, the increase of calcium with ossification and its independence of maternal diet have been examined by Booher & Hansmann, while the relation of the composition of the milk to the composition of the newborn foetus especially as to iron (Bunge's rule) has been studied in some detail for domestic animals by Radeff; Lintzel & Radeff; Adler & Adler; and Belle. The last-named paper, more theoretical than the others, contains interesting double-logarithmic plots. The potassium/calcium ratio in relation to growth-rate is considered by Kaufman & Laskovski, but their data seem too erratic for firm generalisation. Calcium utilisation by developing amphibia has been studied by Luciani, Filomeni & Severi.

Of the individual metals, most attention has been paid to copper. Loeschke has traced most of the copper of the hen's egg to the liver at hatching; Nitzescu has found large amounts in mammalian foetal livers; Sheldon & Ramage have observed spectrographically more copper in all foetal tissues than in the adult; and in insect eggs Melvin has found copper.

HORMONES

The two most important papers in this field are (a) the review on endocrine correlation by Aron, summarising much previous work, and (b) a more speculative review by F. R. Lillie on growth-rate and hormone threshold with reference to the physiology of development. Specifically for adrenalin, there have been papers by Harman & Derbyshire; Cattaneo (1) and Ohnishi on suprarenal development, while the action of the hormone on embryo cells *in vivo* and *vitro* has been tried by Katsuki; Kobayashi; Brinley; Kirihaara; Markowitz; and Matsumori. Specifically for thyroxin, there have been papers by Gudernatsch and Hykes, and Carter has discovered in echinoderm eggs a substance which appears to be similar to this hormone. For pituitrin, see a paper by Küstner. For the sex-hormones, Landauer & Landauer have established a preferential mortality of male chick embryos (indicating presence of sex-hormones), but Louvier finds no difference in size of spur of hen and cock at hatching (indicating their absence). Information on general endocrine development in chondrodystrophic and normal chick embryos will be found in Sun, and a discussion of endocrine aspects of recapitulation theory in Thomson.

VITAMINS

The excellent paper of Adamstone gives particulars of the development of the chick embryo under lack of vitamin E, and the resulting remarkable "lethal ring" which arises at the inner border of the *area opaca* and occludes the extra-embryonic vitelline circulation. A parallel paper is that of Urner, who found the development of the rat embryo to be normal up to the tenth day, after which failure of the haemopoietic tissues occurred. But the most remarkable finding is that of Hill & Burdett, who could show a very high vitamin-E content of the "royal jelly" of bees, the food of the developing larva destined to be queen. Avian hatchability and vitamin E is considered by Card, Mitchell & Hamilton.

The transmission of vitamin A from mammalian parent to foetus has been found by Dann to occur in colostrum and milk rather than through the placenta.

PIGMENTS

With regard to the pyrrol pigments, Lemberg studied oöcyan, the blue-green pigment of many avian egg-shells, and has suggested

a tripyrrol structure. In conjunction with Barcroft & Keilin, he then studied the green pigment of the dog's placenta (now named "Uteroverdin") and found it to consist of an open tetrapyrrol chain. The properties of the two pigments are very similar. As for haemoglobin, its origin has been studied in herring embryos by John, and in amphibian embryos by Slonimski; while the formation of bilirubin in spleen explants has been investigated by Sümegi & Csaba. Such work might throw light on *icterus neonatorum*, the latest review of which is by Anselmino & Hoffmann (1).¹

With regard to the carotinoids, workers interested in vitamin A have been active; thus von Euler & Klussmann have examined the xanthophyll content of human placenta and avian egg yolk, while Virgin & Klussmann have investigated the vitamin A of the latter after the placing of the laying hens on carotinoid-free diet. A general review of yolk-pigments has been published by Mattikov and technical details of estimation are afforded by Terenyi.

With regard to the melanins, Peck and Thumann describe their appearance in mammalian foetal life, and Makarov in the chick's retina. Carmichael thinks the relative sensitivity of slug embryos to ultra-violet radiation is connected with their melanin pigmentation.

Egg-pigments of unknown constitution are described by Duce (silkworm egg), Baumberger & Michaelis (gephyrean egg), and Teissier (cladoceran egg).

PLACENTAL PERMEABILITY AND PLACENTAL METABOLISM

The first biochemical examination of the "placenta" of the selachian fishes has been reported in a brilliant paper of Ranzi's. By a comparison of the degree of cleidoicity of oviparous, ooviviparous, and viviparous selachian eggs, he has shown that the two latter classes depend more on the maternal fluids than the former class does on the sea-water, and that under certain, as yet unknown, conditions, the mere development *in utero* may be as efficient as the elaboration of a definite placental attachment. These facts are illustrated in the accompanying table.

¹ See also Haselhorst & Stromberger (1) on this subject, which is connected with the theory now gaining support that the well-known increase of erythrocytes, haemoglobin, blood volume, etc., toward the end of foetal development, together with a shift of the dissociation curve of the foetal blood, may be reactions adaptive to anoxaemia and parallel with those found at high altitude. Acclimatisation to life *in utero* may be a necessary preliminary to birth.

	Absorbed by Egg from Environment		Value of Organic Substance Gained or Lost from Egg
	Percentage of the Final Value	Water Ash	
Oviparous :			
<i>Scylium canicula</i>	66	74	- 21
Ovoviviparous (i.e., no placenta) :			
<i>Acanthias blainvillei</i>	50	72	- 54
<i>Centrophorus granulosus</i>	50	75	- 22
<i>Scymnus licha</i>	67	58	- 23
<i>Torpedo ocellata</i>	68	66	- 32
<i>Mustelus vulgaris</i>	96	96	+ 350
<i>Trygon violacea</i>	99	99	+1400
Viviparous (with a placenta) :			
<i>Mustelus laevis</i>	98	98	+1060

The loss of organic substance due to combustions, therefore, is in the more efficient cases of reliance on the maternal fish masked by an enormous positive balance of building materials.

Turning to the mammalian placenta, Lell, Liber & Snyder have made a pioneer study of the change in permeability (rabbit) during development. On the twenty-second day 30 per cent of an injected quantity of phenol-sulphonephthalein is excreted via the placenta and 4 per cent through the chorionic blood-vessels; on the twenty-ninth day 15 per cent through the placenta and 40 per cent through the other vessels. With regard to nutritive molecules, both Schlossmann (2) and Naeslund show that the passage of amino-acids through the placenta permits of interpretation as a pure diffusion. Bickenbach & Rupp (1), by feeding the methyl and amyl esters of oleic and palmitic acids (which can be hydrolysed but not synthesised in the body), could show that these esters appeared in the foetal blood, and concluded that the normal passage of triglycerides occurs without chemical change. The same authors (2) have also shown that if fat of very high iodine value is fed, the iodine value of the foetal fat quickly rises to equal that of the maternal dépôts. New data on the phosphorus partition between maternal and foetal blood are given by Timpe, on that of catalase and glutathione by Anselmino & Hoffmann (2), on that of blood-sugar by Nevinny & Schretter. The placenta has been found permeable to adrenalin [Schlossman (1), goat; Cattaneo (3), rabbit]; insulin (Corey, rat); pituitrin [Cattaneo (3), rabbit]; ovalbumin (?) (Natan-Larrier & Richard, guinea-pig); cestode antitoxin (Miller, rat). It has been found impermeable to milk protein (Natan-Larrier & Grimard-Richard, guinea-pig), bacteriophage (Natan-Larrier, Eliava & Rich-

ard, guinea-pig), and colloidal thorium (Menville & Ane, rat). Particularly interesting is the finding of Natan-Larrier, Noyer & Richard, that horse serum protein will pass the guinea-pig placenta if sodium oleate is first injected.

The respiratory metabolism of the placenta itself has been investigated by Loeser, who in an elaborate work finds a high anaerobic glycolysis rate, diminishing with age. There is also significantly a small aerobic glycolysis, which can be abolished by insulin. Normally, therefore, the maternal blood steadily carries away lactic acid from the foetal system. Histochemical details concerning placental fat may be found in Hilgenberg; Sesler; and Yamaguchi.

On the amniotic liquid see, for volume relations, Lell; for hormones, Huddleston & Whitehead; for dyes, Hayashi; and for carbohydrates, Mohs.

HATCHING AND BIRTH

Hatching enzymes [cf. Needham (1), Sec. 24.2] have attracted much attention, e.g., Onorato & Stunkard for parasitic trematodes; Wintrebret & Ouang, and Ouang for selachian and plagiostome fishes; Grandori for the silkworm; Roberts for weevils; and above all Berrill, in an extensive study, for ascidians.

As regards mammalian birth, Heller & Holtz have brought forward new evidence in favour of the pituitrin theory, but the conclusive demonstration of Allan & Wiles that normal birth (in cats) can take place after complete hypophysectomy must be regarded as a considerable drawback to it. For this reason one or other of the chemical "alarm-clock" theories [Needham (1), p. 1609] may again win favour, concerning which see the paper of Strack & Loeschke on the choline in the placenta and the uterine muscle.

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THE COMPARATIVE BIOCHEMISTRY OF VERTEBRATES AND INVERTEBRATES*

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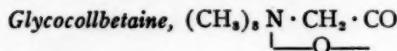
The farther research in physiological chemistry advances the clearer becomes the striking affinity which exists in the chemical composition of the tissues and tissue products of the entire organic world. Inspection by the methods of physiological chemistry reveals increasing similarities, not only within the members of individual plant and animal classes but between animals and plants themselves. Not only are the larger molecules—proteins, fats, lipoids, etc.—quite similarly constituted in all organisms, but even among the numerous substances of smaller molecular size there is to be found an ever-increasing number which are not restricted to a single class of organisms but which possess a much wider distribution than was originally assumed.

This is illustrated by investigation of the various animal phyla for simple bases. Such studies were originally undertaken with the thought that the discovery of simple nitrogenous substances in the different animals would give a deeper insight into intermediary protein metabolism or would possibly extend our knowledge of animal poisons. There resulted, of their own accord, viewpoints of a comparative biochemical nature so that it appears proper to speak in this case of a comparative physiological chemistry.

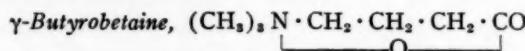
There has been no review of the subject since 1926 (1). It is therefore appropriate not only to consider the pertinent literature of the last year but to include that which has appeared since 1926. This is all the more permissible since it is not very voluminous. Progress in this field can proceed but slowly, for only the isolation of the bases in pure form and their accurate analytical identification are able to really widen our knowledge. How difficult this is, especially with animal materials as the source, is known to everyone who has worked in this field.

* Received November 1, 1932. This review is confined to the simpler nitrogenous bases.

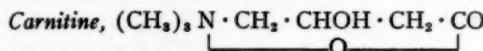
BETAINES



This base has been newly discovered by Okuda (2) in the crustacean *Palinurus japonicus* (2.4 gm. in 1 kg. of fresh flesh) and in the cephalopod *Loligo breskeli* (1.7 gm. in 1 kg. of fresh flesh). It has also been found by Morizawa (3) as well as Iseki (4) in *Octopus octopodia* (68 gm. of almost pure betaine in 36 kg. of octopus muscle), by Hoppe-Seyler and W. Schmidt (5) in the teleost *Pleuronectes cynoglossus*, and by Kutscher and Ackermann (6) in the mussel *Arca noae*.



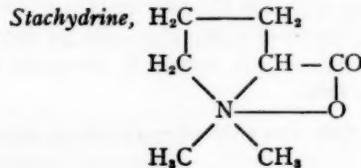
This has been demonstrated by Ackermann (7) to be present in the anthozoan *Actinia equina*, by Hoppe-Seyler and W. Schmidt (8) in *Anguilla vulgaris*, by Kutscher and Ackermann (6) in the mussel, *Arca noae* and by Keil, W. Linneweh, and Poller (9) in the snake, *Python molurus*. In all cases identification was made through the chloride, chloro-aurate, and chloroplatinate.



Carnitine, the betaine of γ -amino- β -hydroxybutyric acid, has been described by Morizawa (3) in the cephalopod, *Octopus octopodia* (platinum salt and gold salt).

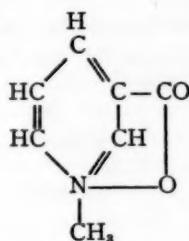


This, according to W. Linneweh (10), denotes a base which he obtained in small quantity from Liebig's extract of beef muscle. The structure has been proven by synthesis.



This base, often observed in plants, was recently found for the first time in the animal world by Kutscher and Ackermann (6). It was isolated from the mussel, *Arca noae*, as the chloride and chloroaurate.

Trigonelline,



Trigonelline, the methyl betaine of nicotinic acid, is likewise a commonly occurring plant alkaloid. Its presence in animals was first reported by Holtz, Kutscher, and Thielmann (11), in the sea-urchin *Arbacia pustulosa*. Haurowitz and Waelsch (12) showed that it probably occurs in the coelenterate *Velella spirans* (jellyfish).

Especially interesting is the discovery of W. Linneweh and Reinwein (13) that trigonelline occurs in normal human urine and is probably identical with the urinary base, gynesin, formerly described by Kutscher. It is also shown that even under complete abstinence from coffee, tea, cocoa, and nicotine, which could cause an excretion of the base, the urine still contains trigonelline (14) so that it can also be formed, perhaps, by the vertebrate organism. Accordingly our former tabulated summary would have to be completed as shown in Table I (p. 358).

As for the origin of the betaines in living things the initial hypothesis that, at least for glycocollobetaine, choline might be regarded as the mother-substance was soon abandoned since other betaines were found which in no case could arise from phosphatides. Engeland (15) was the first to show that all of them, most probably, were derived from the amino acids of proteins, and that several of the most common were none other than methylation products of these amino acids themselves. Schulze and Trier (16), on the basis of their observations, supported this conclusion. Nowadays it is generally accepted as correct. According to it the betaines appear, in some

degree, to be by-products of protein synthesis. They form, primarily, in zones of rapid breakdown or synthesis of protein by the thorough methylation of several of the more resistant amino acids

TABLE I
THE DISTRIBUTION OF BETAINES IN THE ANIMAL KINGDOM

Phylum or Class	Glycocollyl- betaine	Other Betaines and Nitrogenous Bases Present
<i>Vertebrates</i>		
Mammalia	-	γ -butyrobetaine; <i>N</i> -methyl pyridine; carnitine
Reptilia	-	γ -butyrobetaine
Amphibia	-	
Pisces :		
Teleostei	+	γ -butyrobetaine
Chondrostei	+	trimethylamine oxide
Selachii	+	(only in salt-water fish)
Cyclostomata	+	
<i>Invertebrates</i>		
Arthropoda:		
Insecta	-	
Crustacea	+	Trimethylamine oxide; <i>N</i> -methyl pyridine
Mollusca:		
Pelecypoda (mussels) ..	+	γ -butyrobetaine; stachydrine; <i>N</i> -methyl pyridine
Cephalopoda	+	Carnitine; trimethylamine oxide
Annelida	+	
Platyhelminthes	+	
Echinodermata:		
Holothuroidea	+	
Echinoidea	-	Trigonelline
Coelenterata:		
Hydrozoa	-	Trigonelline (?)
Anthozoa	-	γ -butyrobetaine; <i>N</i> -methyl pyridine
Porifera	+	

or their transformation products. The less rapidly combustion and perhaps also exchange of metabolites proceeds, the more readily these compounds form. According to prevailing opinion the processes are much the same, only quantitatively much more extensive, as those which lead to the formation of high-molecular alkaloids in many plants (17).

Whether the formation of such compounds should be regarded as a means of nitrogen-sparing or whether the substances are merely metabolic products of an especially resistant nature which are eliminated later on has been doubtful up to now. Recently, however, the question has received explanation in the studies of Hoppe-Seyler and Linneweh (18) to the effect that the betaines in the animal body and probably in the plant also are to be looked upon as typical excretion products. The authors succeeded in demonstrating the presence of glycocollobetaine regularly and in identifying it analytically through the chloride and chloro-aurate, not only in the blood of *Octopus vulgaris*, but also in the urine collected from different individuals.

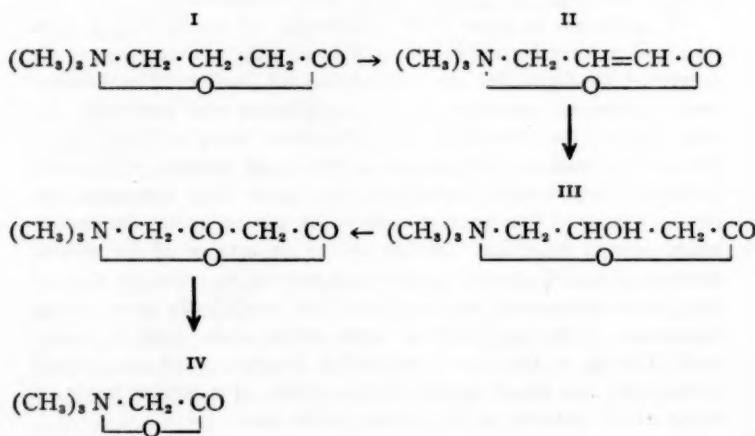
The surprising fact that in the Arthropoda (cf. Table I) glycocollobetaine has not been found up to the present time in insects, although it definitely occurs in crustaceans, can be explained, perhaps, by the rapidity of insect metabolism. Here, glycine can hardly accumulate in appreciable quantity but is further burned and is not available for betaine formation.

While up to the present time a transformation of amino acids into betaines in the animal body has not been directly observed, Klein and Linser (19) have recently attempted to achieve this with plants. They believe there was an increase in the stachydrine content of *Stachys* and *Galeopsis ochroleuca* and in the trigonelline content of trigonella species and dahlias, when certain amino acids were given. Injection into the hollow stems was most favorable. Besides proline, ornithine and glutamic acid were administered.

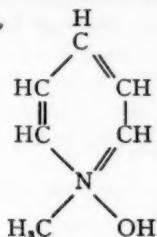
A significant increase in the production of stachydrine is even supposed to have been obtained with hexamethylenetetramine as the formaldehyde-depot for the methylation of proline. The betaines were determined quantitatively by precipitation with potassium bismuth iodide, the bismuth in the precipitate being estimated by a photometric method. On account of the small amounts of betaines concerned, the authors heretofore have made their determinations only by means of this indirect method. In our judgment the experiments deserve repetition. In view of the importance of the results, however, it would appear urgently necessary to so refine the method that direct increases in the weights of the analytically pure betaine compounds, following "feeding" with amino acids, could be measured. Owing to the close relationship between plant and animal metabolism, one should weigh the possibility of a similar mode of origin of the betaines in the animal world also.

The feeding of plants with nicotinic acid in order to obtain trigonelline (cf. formula above) has been postponed by Klein and Linser since they have not yet worked out a procedure for the certain separation of nicotinic acid and trigonelline. The conversion of nicotinic acid into trigonelline in the animal organism, as first demonstrated by Ackermann in 1912, has recently been confirmed by Komori and Sendju (22). As for this conversion in the plant, it is known that in one and the same species, namely in rice, both nicotinic acid (23, 24, 25) and trigonelline (26) are found. The observations of Linneweh and Reinwein (13, 14), as mentioned above, leave open the possibility of trigonelline formation in the vertebrate organism. Since the pyridine nucleus has never yet been observed in the latter, its formation from proline could also, perhaps, be taken into consideration.

A new possibility for the formation of glyccollbetaine in the animal body has been discussed by W. Linneweh (20). He traces the origin of glyccollbetaine from γ -butyrobetaine (I), which has already been found in five different classes in the animal world (cf. Table I). By β -oxidation, according to Knoop (21), this could be catabolized finally to glyccollbetaine (IV) through crotonbetaine (II) and carnitine (III). His success in transforming γ -butyrobetaine and crotonbetaine into carnitine in the mammalian body provides a certain measure of support for this hypothesis.



OTHER METHYLATED NITROGEN DERIVATIVES

N-methyl-pyridine,

N-methyl-pyridine (methyl pyridyl ammonium hydroxide), the occurrence of which in crabs, mussels, and actinzoa has already been described, has again been found by Linneweh and Reinwein (13, 14) as a constituent of normal human urine, in confirmation of an earlier report by Kutscher. Methyl pyridine must be connected with trigonelline, even if its origin from the latter is rendered improbable by the observation that the feeding of trigonelline to cats and rabbits (27) did not lead to the formation of methyl-pyridine.

Trimethylamine oxide, (CH₃)₃N = O

Concerning this base there is much of importance to be added since our last review. Hoppe-Seyler (28) has given attention to "kanirin," a base which Suzuki, Inuye, and Bharatkar (29) obtained from Japanese giant crabs and Suzuki and Okuda (30) from the tai (*Pagrus major*). He proved that in this case it must be trimethylamine oxide, so that this base has now been found not only in the fishes and cephalopods, but also in the crustacea. Poller and Linneweh (31) found trimethylamine oxide in *Clupea harengus*, and Hoppe-Seyler and W. Schmidt (32) observed it in all sea-fish hitherto examined, but not in any fresh-water fish. These latter investigations were even extended to trimethylamine (33), whereby it was demonstrated that the muscle of salt-water fish, quite freshly employed, always contained traces of this volatile base, though it could never be found in the muscles of fresh-water fish (cf. Table II, p. 362).

The quantity of trimethylamine increases, the longer the period after the death of the animal, since the conversion of trimethylamine oxide into trimethylamine is caused by bacterial enzymes. This, incidentally, became known when trimethylamine oxide was first dis-

TABLE II

THE DISTRIBUTION OF TRIMETHYLAMINE AND TRIMETHYLAMINE OXIDE IN FISH

Salt-water Fishes	Trimethylamine	Trimethylamine oxide
Selachii:		
<i>Lamna cornubica</i>	+	+
<i>Acanthias vulgaris</i>	+	+
Teleostei:		
<i>Clupea harengus</i>	+	+
<i>Gadus aeglefinus</i>	+	+
<i>Gadus morrhua</i>	+	+
<i>Mullus barbatus</i>	+	+
<i>Pleuronectes cynoglossus</i>	+	+
<i>Conger vulgaris</i>	+	+
Fresh-water Fishes		
<i>Anguilla vulgaris</i>	-	-
<i>Salmo salar</i>	-	-
<i>Squalius cephalus</i>	-	-
<i>Alburnus lucidus</i>	-	-
<i>Perca fluviatilis</i>	-	-
<i>Cyprinus carpio</i>	-	-

covered in nature by Suwa of the Marburg laboratory. Through these facts it has become clear that the characteristic odor of salt-water fish is due only to the decomposition of trimethylamine oxide, which is common to these fish in contradistinction to fresh-water fish. It was also found by Hoppe-Seyler (34) not only in the muscle but also in the blood and urine of selachians by the isolation and rigorous analysis of several of its characteristic salts, exactly as in all cases mentioned up to the present time. A special procedure perfected for this purpose (reduction with zinc chloride in hydrochloric acid and titration of the distilled trimethylamine) permitted, further, its quantitative measurement. Hoppe-Seyler (34, 35) comes then to the conclusion that with respect to its biological significance trimethylamine oxide is related to urea, which it resembles by virtue of its non-poisonous nature, its small molecular weight, and its almost neutral reaction. One must assume that it originates by the methylation and oxidation of the lowest cleavage products of protein, probably from ammonia, and that it belongs to those metabolism products that help to regulate the tissue osmotic pressure of

animals living in the hypertonic medium of sea-water. According to this, the absence of trimethylamine oxide from the fresh-water teleosts would be understood by their existence in a hypotonic medium. In the urine of marine teleosts trimethylamine oxide has already been convincingly demonstrated by Grollman (36). Kapeller-Adler and Krael (37) later found in the codfish 0.0167 gm. of trimethylamine oxide per 100 gm. of muscle.

Choline and neosine

Choline and neosine have been found by Flössner and Kutscher (38) in the liver of *Raja clavata* (selachian).

GUANIDINE DERIVATIVES

The principle of the alternative occurrence of creatine (creatinine) in vertebrates and arginine in invertebrates, which was first enunciated by Kutscher in 1914 and which, in effect, states that these substances mutually replace each other in these two groups of animals, has been supported by further investigation. The facts pertaining to the occurrence of arginine can be summarized as in Table III.

TABLE III
THE DISTRIBUTION OF ARGININE

	Phylum and Species	Salt of Arginine Analyzed
Arthropoda:		
<i>Melolontha vulgaris</i>		Copper nitrate; nitrate
<i>Crango vulgaris</i>		Copper nitrate
<i>Astacus fluviatilis</i>		Copper nitrate
Mollusca:		
<i>Mytilus edulis</i>		Copper nitrate; nitrate
<i>Arca noae</i>		Nitrate
<i>Eledone moschata</i>		Copper nitrate; nitrate
Annelida:		
<i>Lumbricus terrestris</i>		Copper nitrate
Echinodermata:		
<i>Arbacia pustulosa</i>		Copper nitrate
<i>Holothuria tubulosa</i>		Copper nitrate
Coelenterata:		
<i>Geodia gygas</i>		[Guanidine and agmatine were found in place of arginine]

Certain objections have now been made against the full validity of this principle. Here it should be observed that in case there occurred a small amount of arginine along with much muscle creatine in vertebrates it would only signify that this compound, which in invertebrates occupies such a dominant or exclusive position, is here formed in small quantities, perhaps only as a by-product. This would not be surprising since it is very probable that arginine is also the precursor of creatine in vertebrates, even though such a transformation has not been demonstrated with certainty up to the present time. Besides, arginine is to be regarded as a metabolism product appearing in all organs. Furthermore it must be clearly understood that even if, on the other hand, it could be shown that creatine (creatinine) occurs in small amounts in the invertebrates, the fact would still remain that arginine above all is formed even as in the vertebrates creatine occurs. So that both compounds, in fact, occur alternately.

As is shown by the preceding table, we have, in every case, proved the presence of arginine in the different animal classes by the isolation and analysis of one or more of its salts. One will admit that a complete guaranty for the occurrence of a substance in so complicated a mixture as an animal-organ extract cannot otherwise be given. It would therefore be desirable if the interesting findings of Kiech, Luck, and Smith (40), who, by an indirect method,¹ discovered arginine in rat muscle, would be further supported by the preparative isolation of pure arginine. An experiment recently undertaken by us of obtaining arginine from horse muscle led to a negative result (39).

A communication by Morizawa (3) is, furthermore, worthy of note. He states that arginine could not be found in *Octopus octopodia* but that creatinine was identified by color reactions and analysis of the zinc-chloride double salt. Iseki (4), who recently examined octopus muscle, did not mention the isolation of creatinine and is of the opinion that arginine is here replaced by methyl agmatine. The analysis of a picrate and a positive Sakaguchi reaction for arginine led him to this conclusion. For later experiments it should perhaps be mentioned that betaine, which occurs abundantly in the octopus,

¹ Hydrolysis of arginine by arginase to ornithine and urea, and estimation of the latter by oxidation of its dixanthrydyl compound with potassium dichromate and sulphuric acid.

also gives a good crystalline zinc-chloride double salt [Linneweh (41)], and that the Weyl and Jaffé reactions may be given by many biological substances other than creatine and creatinine.

Okuda (2) found in a kilo of fresh flesh of the crustacean *Palinurus japonicus* 5.2 gm. of arginine and no creatine (creatinine); in the cephalopods he speaks only of a very small quantity. As is to be expected, Haurowitz and Waelsch (12) could not find creatine (creatinine) in the jellyfish, *Velella spirans*, in *Actinia equina* (42), and *Holothuria tubulosa* (42).

With regard to the creatine (creatinine) content of vertebrates an exhaustive study of fishes has been made by Hunter (43). The creatine content of the skeletal muscle of fish varies considerably, not only in different species but also among different individuals of the same kind. However, certain characteristic differences of a quantitative nature exist in the various species, although they are not uniformly strongly pronounced in the teleosts and elasmobranchs. In general the latter have in heart muscle a lower creatine content than the former. On the whole, fish muscle contains more creatine than mammalian muscle. In the heart muscle and testes, mammals have a higher creatine content than fish. The brain, in both cases, contains about the same amount. In fish as well as in birds and mammals, red muscle contains less creatine than white, and foetal muscle less than that of the adult.

Creatine (creatinine) was found in snake muscle by Keil, Linneweh, and Poller (9).

An interesting application of the principle of the alternative occurrence of arginine and creatine (creatinine) in invertebrates and vertebrates was undertaken recently by Flössner (44). He sought to answer the question whether animal phyla which morphologically as well as ontogenetically reach a certain stage also show changes in metabolism and chemical structure. He investigated tunicates. One is accustomed to regard these as the last class of the annelids, although they deviate very considerably in structure and appearance from these creatures. Formerly, on account of their outward resemblance to mussels, they were classified with the mollusks. The ontogenetical agreement has led, however, to a reunion of the vertebrates and tunicates as chordates, and the tunicates on account of their morphological relations are to be regarded as the bridge or connecting link. The investigations of Flössner on *Ciona intestinalis* and *Phallusia mammilata* led to the discovery that these animals fail

to contain creatine (as well as methylguanidine and carnosine), while arginine² (and histidine) even though present in small amounts could be demonstrated. In respect to their metabolism, the tunicates are therefore more closely related to the invertebrates than to the vertebrates.

The question of the alternative occurrence of arginine and creatine in invertebrates and vertebrates has awakened renewed interest since it has been observed that both of these bases are associated in organic composition with phosphoric acid in the respective muscles. As phosphagen, these compounds were first described by Fiske and Subbarow (45), as well as by Eggleton and Eggleton (46) (creatine-phosphoric acid) and further by Meyerhof and Lohmann (47) (arginine-phosphoric acid). It was rightly pointed out (48) as a confirmation of Kutscher's assertion. Recently, by an indirect method of analysis, Needham, Needham, Baldwin, and Yudkin (49) have made comparative physiological studies of both phosphagens with the results, up to the present time, summarized in Table IV.

TABLE IV
THE DISTRIBUTION OF ARGININE PHOSPHATE

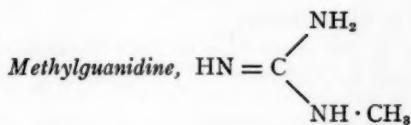
Phylum or Class	Genus	Portion Analyzed
No arginine phosphate:		
Coelenterata	Anthea	Tentacles and body wall
Arginine phosphate alone:		
Coelenterata	Pleurobrachia	Whole body
Platyhelminthes	Planaria	Whole body
Platyhelminthes	Polycilis	Whole body
Nemertinea	Lineus	Whole body
Annelida	Sabellaria	Whole body
Annelida	Spirographis	Whole body
Annelida	Nereis	Whole body
Podaxonia	Sipunculus	Body wall
Cephalopoda	Sepia	Fin, funnel, mantle, and tentacle muscle
Cephalopoda	Octopus	Fin, funnel, mantle, and tentacle muscle
Echinodermata	Synapta	Body wall
Echinodermata	Asterias	Tube-feet
Urochorda	Ascidia	Muscle

² Identified by the melting-point of the copper-nitrate salt.

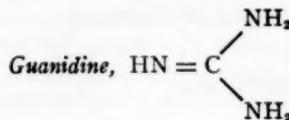
TABLE IV (*Continued*)

Phylum or Class	Genus	Portion Analyzed
Creatine phosphate and arginine phosphate together:		
Echinodermata	Strongylocentrotus	Jaw muscle
Hemichordata	Balanoglossus	Whole body
Creatine phosphate alone:		
Cephalochordata	Amphioxus	Whole body
Craniata	Many different species	

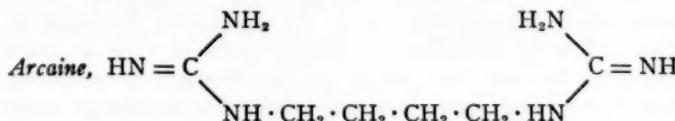
Like Flössner (44), Needham and his collaborators have given special attention to those animals which are regarded as connecting links between the invertebrates and the vertebrates. It is of interest that they found both phosphocreatine and phosphoarginine in the hemichordate, *Balanoglossus*, and in the echinoderm, *Strongylocentrotus*. From an authoritative zoological point of view a certain relationship between these animals and the chordates is recognized. From the chemical point of view, a relationship accordingly exists with the invertebrates as well as with the vertebrates. At any rate it may be seen that intensive investigation in comparative biochemistry presents a possibility of confirming or extending present theories of animal phylogenesis, and it is to be hoped that by the further pursuit of this work many new facts may be brought to light which will be of importance to systematic zoölogy.



We have already pointed out (1) that this base is restricted to the vertebrates (probably on account of its close relationship to creatine). Within recent years it has never been found in invertebrates, although it has been observed several times in vertebrates. Thus its presence in snake muscle has been established by Keil, Linneweh, and Poller (8) and in teleosts by Okuda (50).

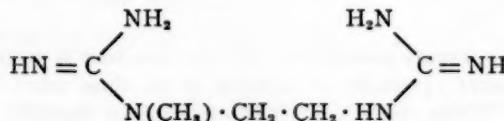


Guanidine was identified by Morizawa (3) in *Octopus octopodia* by the nitrogen content and decomposition temperature of a picrate from the arginine-histidine fraction. At the same time Okuda (50) described in octopus muscle, also as a constituent of the arginine-histidine fraction, a base called octopin, $\text{C}_9\text{H}_{18}\text{N}_4\text{O}_4$, of which the picrate and picronolate were analyzed. In hydrochloric-acid solution it was strongly dextrorotatory and contained no amino nitrogen according to the Van Slyke method. The four nitrogen atoms are suggestive here, also, of a guanidine derivative.



This base which until now had not been described either in the plant or animal world was discovered by Kutscher, Ackermann, and Flössner (51) in the mussel *Arca noae*. Its structure, as determined by Kutscher, Ackermann, and Hoppe-Seyler (52), was confirmed by comparison with the synthetic compound prepared according to Kiesel's method (53). Since the substance was again found without difficulty in another consignment of mussels (54) which were newly investigated, it is clear that it is not an exceptional but a regular metabolic product of these animals.

The possibility of the occurrence of such diguanidines has already been discussed by Kutscher (55) in proposing the formula



for vitiatin, isolated by him from human urine. On account of the shortage of material and especially because the invaluable help of

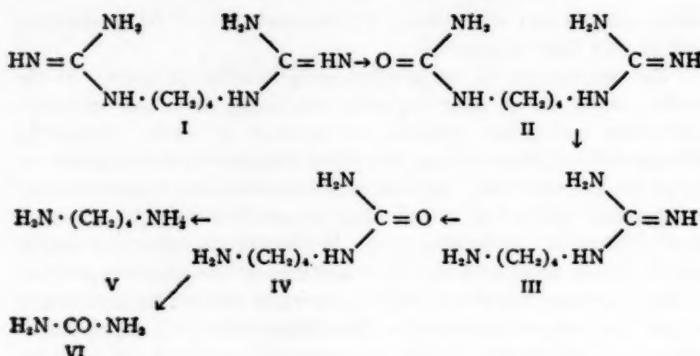
micro-analysis was still lacking, the establishment of its constitution was at that time impossible.

The appearance of tetramethylenediguanidine (arcaine) in the animal world is, in some respects, surprising since this nitrogen-containing metabolism product, in contrast to others, cannot be directly derived from the amino acids of the protein molecule known up to the present time. Of biological importance is its relationship to agmatine, which has already been observed in the lower animals by F. Holtz (56) in *Geodia gygas*. Without doubt agmatine can be easily derived from arginine. The addition of the second guanidine nucleus as it appears in arcaine is at present difficult to understand unless one accepts a synthesis. The supposition of a diguanidino-valerianic acid as the mother-substance of arcaine is obvious, as Zervas and Bergmann (57) have pointed out. On the other hand its formation by the disproportionate conversion of two molecules of agmatine into one molecule of arcaine and one of putrescine may be postulated. Nevertheless we believe that the problem of the origin and fate of arcaine can best be solved through the fortunate discovery of new, related, guanidine derivatives in living nature.

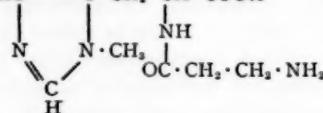
Arcaine is a guanidine derivative of marked pharmacological activity and has been examined in this respect by W. Linneweh (58). It is very similar in structure to the artificially prepared diguanid, synthalin (decamethylenediguanidine) (59). Like it, it diminishes the blood-sugar content and lowers the blood-pressure. It is about twice as poisonous as guanidine and in equivalent doses shows the cramps typical of guanidine poisoning. Arcaine can therefore be classed as one of the few, well-known, synthetically produced, animal poisons. Another is tetramethylammonium hydroxide, which acts like curare and has been observed, up to the present time, only in *Actinia equina* by Ackermann, Holtz, and Reinwein (76, 77).

The fact that this poisonous substance was found in a mussel raises the question as to whether arcaine could perhaps play a rôle in bringing about the well-known mussel-poisoning. However, mussel-poisoning is quite different in nature from arcaine-poisoning. Furthermore, we were unable to demonstrate arcaine in *Mytilus edulis* (54).

According to the studies of F. Linneweh (60) the bacterial decomposition of arcaine (I) leads to carbamyl agmatine (II), agmatine (III), carbamyl putrescine (IV), putrescine (V), and urea (VI).



IMIDAZOLE DERIVATIVES

Anserine, $\text{HC}=\text{C} \cdot \text{CH}_2 \cdot \text{CH} \cdot \text{COOH}$ 

Anserine is carnosine with one nitrogen atom of the imidazole nucleus carrying a methyl group. It was first observed by Ackermann, Timpe, and Poller (61, 62) in goose muscle. Comparison of its cleavage products with synthetic compounds by Linneweh, Keil, and Hoppe-Seyler certified to the correctness of the foregoing formula (63, 64, 65). Later it was found by Hoppe-Seyler, W. Linneweh, and F. Linneweh (66) in the muscles of other species of birds and finally in several reptiles. At the same time, in continuance of an investigation by Keil, W. Linneweh, and Poller, the presence of carnosine was demonstrated. The results may be summarized as follows:

<i>Birds</i>	<i>Anserine</i>	<i>Carnosine</i>
Goose, chicken, turkey, pigeon, crow.....	+	?
<i>Reptiles</i>		
Crocodile	+	+
Snake	-	+

Later the occurrence of anserine in chicken flesh was confirmed by Tolkatschewskaja (67). Ackermann and Hoppe-Seyler (68)

have, furthermore, reported on the muscle extracts of a selachian and of several teleosts which were examined for carnosine and anserine. Clifford (69), also, has published data pertaining to the occurrence of carnosine in these animals. She investigated the muscle extracts of teleosts, colorimetrically, using a diazo method with the altogether doubtful assumption that about 95 per cent of the substances which give the Pauly diazo reaction would be present as carnosine (70). She came to the conclusion that carnosine regularly occurred in considerable quantity in a large number of teleosts, except in the sub-order Anacanthini, that is to say in plaice and cod, from which it was entirely lacking. Also in muscle extracts of these animals examined by Dietrich (71) the diazo reaction of the carnosine fraction remained negative.

Previously, Suzuki and co-workers (72) had published a report on the occurrence of carnosine in muscle extracts of tai (a Japanese salt-water fish), tunny, salmon, and river eel. From the data given it does not appear quite certain that, in every case, carnosine and not anserine was concerned since the analytical values of the salts which were prepared as well as their melting-points would hardly be acceptable for this compound. Above all it was expressly stated in one case that the substance reputed to be carnosine gave no diazo reaction. As is well known, anserine in contrast to carnosine does not give Pauly's diazo reaction. The characteristic copper salt of carnosine was obtained by the authors in only one case—from the muscle of the river eel.

Ackermann and Hoppe-Seyler (68) examined muscle extracts of the herring-shark (*Lamna cornubica*) as a representative of the selachians, of the cod (*Gadus morrhua*) as a typical member of that group of teleosts whose muscle according to Clifford contains no carnosine, of the herring (*Clupea harengus*), which according to Clifford forms carnosine, of the sea eel (*Conger vulgaris*), and the fresh-water eel (*Anguilla vulgaris*), and finally of the perch (*Perca fluviatilis*) as a fresh-water teleost in which Clifford demonstrated carnosine. The following table gives a summary of the results.

In the herring-shark, therefore, anserine occurs in large quantity, and carnosine, at the most, in very small amounts which could not be isolated. In the cod and salt-water eel carnosine is completely replaced by anserine, while the fresh-water eel contains only carnosine. From the muscle extracts of the herring and perch neither anserine nor carnosine could be isolated. The positive diazo test

TABLE V

THE DISTRIBUTION OF ANSERINE AND CARNOSINE IN FISH

Species	Results of Clifford (Diazotest)	Results of Ackermann and Hoppe-Seyler Diazotest	Anserine	Carnosine
<i>Lamna cornubica</i>	+ (moderate)	+	-
<i>Gadus morrhua</i>	-	-	+	-
<i>Clupea harengus</i>	+	+	-	-
<i>Conger vulgaris</i>	-	+	-
<i>Anguilla vulgaris</i>	+	+	-	+
<i>Perca fluviatilis</i>	+	+	-	-

suggests the probability that in both at least the latter compound occurs.

The fact that the three kinds of fish in which anserine was found were salt-water species, and especially that the muscle of the salt-water eel contained the methyl-bearing anserine while that of the fresh-water eel contained only the methyl-free carnosine suggests a relationship to trimethylamine-oxide formation. As has been shown before, the salt-water species form trimethylamine oxide, while the others are free of it (32), causing one to inquire as to whether in the formation of anserine the processes called into play are similar to those concerned with trimethylamine oxide. From the circumstance that in the cod and conger eel carnosine is entirely replaced by anserine, it may be assumed that the latter is formed from carnosine by methylation. At the same time the hypothesis that the methylated bases of muscle are derived from protein cleavage products (16, 17) receives further support, since the origin of carnosine (β -alanyl histidine) from the protein molecule can hardly be doubted.

Since anserine has not heretofore been found in ox muscle (66) it might be assumed that this base is lacking from mammals generally (64). In the meantime, however, the recent exhaustive studies of Wolff and Wilson (73) have shown that the musculature of the dog, cat, rabbit, and white rat contains anserine, it being identified as the copper salt, nitrate, and free base. In horse muscle anserine was not found by these authors, although carnosine could still be identified through the characteristic copper salt. The authors say finally:

Our demonstration that muscles of certain mammals contain anserine permits the conclusion that neither anserine nor carnosine is associated exclusively with any particular group of vertebrates. The occurrence of the two com-

pounds suggests that their functions may be more or less interrelated. In general, muscles high in carnosine are low in anserine and vice versa. Accurate comparisons, however, can only be made when more and better data are available. There is some indication that total iminazole content (carnosine plus anserine) may be important for physiological considerations.

Histidine

This was observed by Okuda (2) in very small quantities in the muscles of *Palinurus japonicus* as well as in *Loligo breekeri*, by Flössner and Kutscher (38) in the liver of *Raja clavata*, and by Morizawa (3) in *Octopus octopodia*.

PURINE DERIVATIVES

Here there is little new to be reported. In the flesh of different fish (*Katsuwonus pelamis* and *Auxis tapeinosoma*) Okuda (50) has found xanthine, hypoxanthine, and guanine. In the crustacean, *Palinurus japonicus*, and in the cephalopod, *Loligo*, the same author (2) observed hypoxanthine. Furthermore adenine has been found in reptile muscle (python) by Keil, Linneweh, and Poller (9), and hypoxanthine and xanthine in the jellyfish, *Velella spirans*, by Haurowitz and Waelsch (12). Hoppe-Seyler and W. Schmidt (8) demonstrated that hypoxanthine was present in all of the salt- and fresh-water fish examined by them. From the muscle of *Octopus octopodia* Morizawa (3) isolated guanine, adenine, xanthine, and hypoxanthine. In octopus urine Hoppe-Seyler and W. Linneweh (18) found hypoxanthine.

TAURINE

In confirmation of earlier reports this substance has been shown to be present in *Mytilus edulis* by Daniel and Doran (74) and in octopus muscle by Morizawa (3). Taurine was also found by Okuda (2) in the crustacean, *Palinurus japonicus* (in small quantities only), and in the cephalopod, *Loligo* (1.8 gm. in 1 kg. fresh flesh). Finally, the presence of taurine in the bile of toads was established by Okamura (75) and by Okuda (50) in the muscle of the fish, *Katsuwonus pelamis*.

Taurine is without an asymmetric carbon atom, a property that it shares in common with the majority (4/5) of the simpler bases here mentioned. The others may be listed as follows: glycocollbe-

taine, γ -butyrobetaine, crotonbetaine, trigonelline, methylpyridine, trimethylamine oxide, tetramethyl ammonium hydroxide, choline, creatine, guanidine, methyl guanidine, arcaine, adenine, guanine, xanthine, and hypoxanthine. On the other hand, only the following have an asymmetric carbon atom: stachydrine, carnitine, arginine, carnosine, and anserine.

This property can hardly be incidental and may, perhaps, be understood by reference to the excretory products contained in normal mammalian urine. Almost all of these substances, also, are characterized by the lack of an asymmetric carbon atom, a fact conveying the impression that the organism is especially disposed to excrete, as useless, substances devoid of optical activity. In the light of this hypothesis many of the simpler bases in invertebrates as well as in vertebrates may be regarded essentially as excretory products.

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DETOXICATION MECHANISMS*

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The object of this contribution is to discuss the more recent articles on detoxication, and at the same time to include the more important additions to the literature in this field since the publication ten years ago of an article on detoxication by one of the authors (1). We have followed the same general scheme as outlined in the previous contribution, including oxidation, conjugation under the various subheadings of glycine, glutamine, ornithine, and cystine in the form of mercapturic acid, glycuronic acid, methylation, acetic acid, and sulfuric acid.

OXIDATION

Knoop (2), Dakin (3), and others have shown that aromatic derivatives of the fatty acids are oxidized in the animal body according to the rule of beta oxidation, forming benzoic or phenylacetic acid. Ward (4) found an exception to this rule in indole propionic acid, which was oxidized at the α -carbon atom and excreted as indole acetic acid. Beznak (5) showed that β -phenyl glutamic acid when injected into dogs and rabbits is oxidized both in the α H and the β H position. Flaschenträger (6) tested the oxidation of long-chain fatty acids in order to determine whether oxidation might also take place on the delta and zeta carbon atoms.

Peters & Watanabe (7) fed γ -benzolsulfo-methyl-amino-*n*-valeric acid to dogs and rabbits; they found the substance excreted unchanged. This was in contrast to the phenyl fatty acids where the phenyl group is attached to the end carbon atom, for these compounds undergo oxidation to benzoic acid (here the radical substituted in the phenyl group is attached to the end carbon atom of an α -aminovaleric acid. Oxidation ceases with the formation of a 4-carbon chain).

Keil (8) fed δ -aminovaleric acid to dogs and isolated from the urine 4-amino-2-butanone. Peters (9) fed δ -benzolsulfo-methyl-

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aminovaleric acid and found it oxidized to the corresponding propionic acid. When this substituted propionic was fed, it appeared unchanged in the urine. His conclusion was that the nature of the end-product of the oxidation of a substituted fatty acid is determined by the radicals between the carboxyl and the amino group.

Flaschenträger & Beck (10) fed ζ -benzolsulfo-methyl-amino-heptanoic acid and found that 88 per cent of the amino acid was oxidized to the corresponding propionic acid. Flaschenträger & Halle (11) fed 10-benzolsulfo-methyl-amino-undecanoic acid and found that it was oxidized according to the scheme of beta oxidation and that it was demethylated forming γ -benzolsulfo-amino-valeric acid. Demethylation appears to have been accidental rather than the result of any physiological process. The dicarboxylic acids (12), adipic, suberic, and sebacic, were found much more resistant to oxidation than the monocarboxylic acids. Only the amide of sebacic acid could be traced quantitatively; and this appeared in the urine in the form of allantoin.

Peters (13) fed benzoylated substances such as γ -benzoyl-amino-butyric acid, and found that its fate was similar to the benzolsulfo-amino-butyric acid. The substitution of a hydroxyl group in the β position of the γ -benzol-amino-butyric acid seems to increase rather than to decrease its resistance to oxidation. Δ -benzoyl-ornithine was fed and most of it was recovered from the urine unchanged.

Pelargonic acid (11) is completely burned in the animal body.

Hosoda (14) fed *o*-nitro benzaldehyde and *o*-amino benzaldehyde and found that both were apparently oxidized to the corresponding acid; but there was no reduction of the urine which might have been indicative of a glucuronic acid conjugate.

Nicotinic acid (15) is eliminated in the urine of rabbits as nicotinuric acid, but in the urine of dogs it is eliminated as trigonelline. Phenylsuccinic acid (16) is apparently unoxidized in the animal organism. Phenylacylated derivatives (17) of glycine, alanine, leucine, glutamine, glutamic acid, asparagine, aspartic acid, and ornithine were fed or injected into dogs, rabbits, fowls, and human subjects; these were recovered unchanged in the urine. It appears that ornithine, glycine, or glutamine, which are used for the detoxication of phenylacetic acid in these different subjects, is not formed simply by the breaking down or the building up of a single amino acid.

Quinaldine (18) fed to rabbits is oxidized to α -picoline, forming an acid resembling picolinic acid, which is then conjugated with

glycine. *O*-nitro-cinnamic acid when fed to dogs is partly excreted unchanged and partly as *o*-nitro-hippuric acid, with no evidence of quinoline-ring formation. Quinoline (19) fed to dogs is recovered as methyl-quinolinium hydroxide. Quinoline fed to rabbits gives a partially oxidized product which appears in the urine as an ethereal sulfate. Methyl-quinolinium hydroxide (20) fed to dogs, rabbits, and chickens is found to be less toxic than quinoline and is excreted unchanged in the urine.

Benzylamine (21) fed to dogs gave varying amounts of hippuric acid, while β -indoylethylamine (22) was deaminized and oxidized to β -indolacetic acid. Trimethylamine and monomethylamine (23) were completely metabolized in the organism. Investigation of the fate of aromatic cyanides showed that *p*-nitro-, *p*-chloro- and 2,4-dichlorophenyl cyanide, as well as the *p*-chloro-, *o*-nitro- and *p*-nitro-benzyl cyanides, were to a slight extent hydrolyzed into the corresponding acids; nor did there seem to be a splitting-off of the cyanide group, though there was a decided increase in the output of ethereal sulfates (24). Triphenyl derivatives, such as triphenyl methane, triphenyl carbinol, and triphenyl acetic acid, were excreted largely unchanged by animals (25). Diphenylacetic acid was excreted by humans as well as by animals in combination with glycuronic acid (26).

GLYCINE

Glycine has been studied recently with a view to determining its possible source and method of formation, as well as the influence of certain dietary conditions upon its origin. A great deal of work has also been done in order to determine the possibility of using some simple glycine conjugate as a test for renal or hepatic function.

Sherwin & Shipley (27) studied the synthesis of glycine in the animal organism. A man was placed on a non-protein diet and fed benzoic acid in order to determine whether the body was able to produce relatively large amounts of glycine for the detoxication of the benzoic acid. They showed that a 5-gram dose of benzoic acid could be detoxicated within a period of 12 hours without increasing the total nitrogen output. Their results indicate that glycine is synthesized under these conditions by the human body at the expense of nitrogen which would otherwise have been excreted as urea.

Griffith & Lewis (28) found that the rate of hippuric-acid

excretion in rabbits after feeding benzoic acid was greatly increased by simultaneous administration of glycine. This they believed was due not to more rapid absorption, nor to the specific stimulation of general metabolism by glycine, since alanine had no such effect, but to the presence of preformed glycine. Simultaneous administration of alanine, cystine, leucine, nor-leucine, iso-valine, aspartic acid, glycolic acid, glycol aldehyde, glucose, or urea with sodium benzoate caused no increase in the rate of excretion of hippuric acid; from which the authors concluded that none of these substances are readily available precursors of glycine.

The same authors (29) note that the rate of excretion of hippuric acid following the oral administration of sodium benzoate to rabbits is increased by the simultaneous administration of the products of the peptic-tryptic digestion of elastin and gelatin (which contained much glycine) but not by the administration of similar preparations from peptone, edestin, glutenin, casein, egg albumin, or peanut meal. This failure was thought to be due to no interference in absorption, for administration of glycine with the last-named substance caused the usual increase in hippuric-acid excretion.

Bignami & Boraccia (30) administered 50 grams of benzoic acid within a space of five to six hours and found 45 to 80 per cent eliminated as hippuric acid. They believe that glycine formation in this case must be ascribed to a special metabolism, for this synthesis is associated with a rise in total nitrogen. It is quite impossible to tell in this case how much of the experiment can be counted as physiological or how much of it is to be considered pathological.

Brigl & Pfahler (31) have investigated the reason for the excretion of large amounts of hippuric acid in the urine of herbivora. They believe lignin is the mother-substance of the hippuric acid excreted by rabbits and sheep. They found three times as much of the acid in the urine after feeding hay as after feeding cereal straw.

Widmark & Jensen-Carlen (32) believe that the carbohydrate of the diet has a marked effect upon the production of hippuric acid, inasmuch as persons ingesting a low carbohydrate diet are apt to excrete much less hippuric acid after administration of benzoic acid. Widmark (33) has also announced that he believes an acid-producing diet, such as fat and protein without carbohydrate, causes a very striking diminution in the synthesis of hippuric acid after sodium benzoate feeding, the decrease varying from 9 to 16 per cent during the preliminary period up to 60 per cent on the fourth day of the

experimental period when carbohydrate was lacking in the diet. Further, after a restoration of carbohydrate to the diet the amount of hippuric acid excreted increases markedly.

Delprat & Whipple (34) believe that the liver plays the chief part in the synthesis of hippuric acid, but that other cells of the body can take over this work, if necessary. They find the synthesis of hippuric acid much delayed by severe liver necrosis induced by chloroform.

Morgulis, Pratt & Johr (35) state that in nephritis hippuric-acid synthesis is never complete, but varies between 53 and 95 per cent of the theoretical amount. They base their figures on six-hour periods.

Snapper (36) finds that men with normal kidneys will excrete 5 grams of benzoic in twelve hours in the form of hippuric acid. He believes that the glycine supply of the body for this conjugation is independent of the bile secretion. Snapper & Grünbaum (37) believe that persons with normal kidneys should excrete 5 grams of sodium benzoate in three hours. Patients with lobar pneumonia, chloride retention, or complete obstruction of the bile can excrete hippuric acid to the same extent that normal persons do. In fact, Snapper (38) states that the reduced excretion of hippuric acid in nephritis is not due to failure of the kidneys to synthesize this product, but the failure to excrete it.

Violle (39) believes that the synthesis of hippuric acid is not affected by diseases of the liver, as he finds in all renal disturbances diminished hippuric-acid excretion; he also notes that the amount of its excretion runs parallel to the other renal function test and to albuminuria. In other words, the decrease in hippuric-acid excretion is directly proportional to the kidney damage.

GLUTAMINE

Glutamine has been found as a detoxicating agent only in the human subject (40). It has been isolated in the form of phenylacetyl glutamine or phenylacetyl-glutamine urea after the ingestion of phenylacetic acid. Glutamine, like glycine, can apparently be synthesized from waste nitrogen which would have otherwise appeared in the urine as urea (41). A small amount of phenylacetic acid is always excreted combined with glycuronic acid (42). Most of the derivatives of phenylacetic acid have been fed to human subjects; but thus far none excepting phenylacetic acid itself has been found in combination with glutamine.

FATE OF SOME PHENYLACETIC-ACID DERIVATIVES

Compound Fed	No Conjugation	With Glycine	Acetylated	Reference
<i>o</i> -hydroxyphenylacetic acid.....	Man, dog, rabbit	2
<i>m</i> -hydroxyphenylacetic acid.....	Man, dog, rabbit	3
<i>o</i> -aminophenylacetic acid.....	Man, dog	Rabbit	2
<i>m</i> -aminophenylacetic acid.....	Man, dog	Rabbit	3
<i>p</i> -aminophenylacetic acid.....	Dog	Man, rabbit	1
<i>o</i> -chlorophenylacetic acid.....	Rabbit	Man, dog	2
<i>m</i> -chlorophenylacetic acid	Man, dog, rabbit	3
<i>p</i> -chlorophenylacetic acid.....	Rabbit	Man, dog	1
<i>p</i> -bromophenylacetic acid.....	Rabbit	Man, dog	1
<i>m</i> -nitrophenylacetic acid.....	Man, rabbit	Dog	3
<i>o</i> -nitrophenylacetic acid.....	Man, rabbit, dog, chicken	2
2,4-dinitrophenylacetic acid	Man, dog, rabbit	2
tri-phenylacetic acid	Dog, rabbit	5
di-phenylacetic acid.....	Man, dog, and rabbit with glycuronic acid	4

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ORNITHINE

Ornithine has been found only in the fowl, where it is used as a detoxicating agent to combine with organic acids, such as benzoic acid, forming a di-benzoyl-ornithine. Fowls, so far as we are aware, are unable to use the other detoxicating agents such as glutamine, glycine, glycuronic acid, and sulfuric acid. On account of the difficulty in separating urine from feces, work on the fowl has proceeded slowly.

Crowdle & Sherwin (43), providing chickens with an artificial anus, whereby they were able to separate the urine from the feces, fed them benzoic acid. Ornithuric acid was isolated from the urine. The ornithine for the detoxication of the benzoic acid was synthesized in spite of the fact that the chickens were on a non-nitrogenous diet. The nitrogen for the synthesis of the ornithine appears to have

been taken from the uric-acid fraction. The ingestion of arginine increases the output of ornithine, while other amino acids and even histidine seem to have little effect.

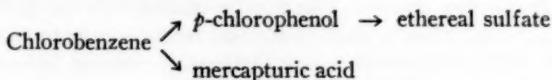
CYSTINE (MERCAPTURIC ACID)

During the last ten years several workers have tried to solve some of the problems of sulfur metabolism by studying the formation of mercapturic acid after the administration of bromobenzene.

Sherwin and co-workers (44 to 48 inclusive), having confirmed the results of Thomas (48a) and Kapfhammer (48b) that on a low-protein or carbohydrate diet no mercapturic acid is formed, devised an experiment calculated to test the animals' ability to synthesize cystine. Dogs were maintained on a carbohydrate diet and received bromobenzene along with various forms of sulfur and nitrogen. Although sodium sulfate, potassium sulfocyanide, calcium sulfide, taurine, ethyl-amino mercaptan, and cystine were fed, mercapturic acid could not be isolated from the urine except when cystine was administered with bromobenzene. Failure to obtain mercapturic acid in all other cases led to two conclusions: (a) that the organism is unable to synthesize cystine from the forms of sulfur and nitrogen fed; (b) that the cystine of body tissue cannot be utilized for detoxication of bromobenzene, even though this latter substance induces tissue catabolism. It was also observed that a large relative increase in ethereal sulfate occurred when bromobenzene was fed on a carbohydrate diet, but that the simultaneous administration of cystine reduced the ethereal sulfate by one-half. This was interpreted as evidence that ethereal sulfates are mainly endogenous in origin, and a theory was proposed that a mercapturic acid was an intermediate product in their formation. The mechanism proposed was as follows: when exogenous cystine is absent, the phenolic compound attaches itself to an intermediary catabolite of tissue cystine, and the conjugated product is then oxidized to ethereal sulfate; when exogenous cystine is present, the phenolic compound attaches itself directly to cystine to form phenyl-mercapturic acid, which is then either eliminated as such or oxidized to sulfate.

Hele and co-workers (49 to 56 inclusive) published several articles during the same years (1924-27) in which some of the findings and conclusions of Sherwin are strenuously controverted. Whereas Sherwin (45, 46) found inorganic sulfates ineffective for the detoxication of phenols, Hele (56) obtained an increase in ethereal sul-

fates by simultaneous administration of phenolic compounds and sodium sulfate, the increase occurring at the expense of the inorganic sulfate fraction in the urine. Also, after feeding iodo-phenyl-mercapturic acid to a pig, Coombs and Hele (54) accounted for 83 per cent of the dose as neutral sulfur in the first 24 hours, and no increase in ethereal sulfate was observed, while Sherwin (45, 46) reported a partial oxidation of this substance to ethereal sulfate. Lawrie (55) reported no oxidation of the substance to ethereal sulfate in the rabbit. In Hele's work, contrary to the findings of Rhode (57) and Sherwin (46), *p*-chlorophenol gave no indication of mercapturic-acid formation; and the sulfur partition in the urine was very different from that produced by chlorobenzene, a result which led him to conclude that *p*-chlorophenol is not an intermediate product in the transformation of chlorobenzene to mercapturic acid, but a distinctly different detoxication reaction. This result is confirmed by the recent work of White and Lewis (58). By an analysis of his sulfur figures, Hele found that a relatively constant ratio exists between the extra ethereal sulfate and extra neutral sulfur, such that $\frac{E}{E + NS} = K 40$, where *E* represents extra ethereal sulfate and *NS* represents extra neutral sulfur. This is taken to mean a balance between two simultaneous reactions occurring in the cell:



The difficulty in interpreting urinary sulfur-partition figures may be largely responsible for the contradictory views formulated in these two laboratories.

Coombs, Callow & Hele (51, 52) made the interesting observations that benzene, *o*- and *m*-dichlorobenzene, and fluorobenzene cause a rise in neutral sulfur, indicating mercapturic-acid formation; but in no case was such a product isolated. Toluene, *o*-chlorotoluene, *o*-, *m*-, and *p*-chloroacetanilide, *o*-, *m*-, and *p*-chloroanisole caused no increase in neutral sulfur.

Recently, Lawrie (55) has reported the isolation of iodophenyl mercapturic acid from the urine of rats and rabbits after injection of iodobenzene; the acid was isolated by the method of adsorption on Merck's charcoal. With rats approximately 8 per cent of the injected iodobenzene was recovered as mercapturic acid, with rabbits slightly

less than 1 per cent. Lewis (59) studied the effect of bromobenzene on sulfur metabolism in the rabbit, and concluded from his sulfur-partition analyses that the rabbit detoxicates bromobenzene as effectively as the dog. Caution must be used in interpreting neutral sulfur figures; and one is inclined to question the validity of assuming that the increase in neutral sulfur represents only mercapturic acid, especially when it indicates such large yields of the acid in fasting rabbits. Lewis pointed out that the small yield in Lawrie's experiment could be ascribed to two factors: (a) the excessively large doses of iodobenzene (3.6 gm. per rabbit in a single dose) which Lewis found to be less effectively detoxicated than smaller doses; (b) the basic diet which was reported by Abderhalden (60) to retard this synthesis. This latter point will be considered presently. The finding of mercapturic acid in rat and rabbit urines definitely answers a question proposed ten years ago as to whether mercapturic acid could be formed by animals other than the dog.

Evidence in favor of this synthesis in the rabbit was presented by Abderhalden & Wertheimer (60, 61) who isolated small quantities of the compound after injection of bromobenzene on a diet of oats; but they obtained no trace of mercapturic acid when a diet of green vegetables was used, thus indicating that the synthesis is favored by an acid diet and inhibited by an alkaline diet. This was taken as another example to substantiate a theory that detoxication processes can be modified or entirely changed by the acid or basic character of the diet. However, it should be mentioned that of several detoxication processes reported to be dependent on this factor, Griffith (62) showed hippuric-acid synthesis to be unrelated to this dietary factor; and Braunstein, Parschin & Chalisowa (63) gave striking evidence to show that the extent of oxidation and detoxication of benzene, toluene, and phenol is dependent on the factor of diuresis, and independent of the acid or basic character of the diet as such. Mercapturic acid seems to be the only outstanding example of a detoxication product whose formation is dependent on the acid or basic character of the diet.

In the author's laboratory the experiments of Abderhalden & Wertheimer on mercapturic acid were repeated, using their technique and method of isolation, and, as these workers found, mercapturic acid was readily isolated from the urine of rabbits on the oat diet, while no trace of the crystalline substance was obtained on the green-food diet. However, it was found that simple chloroform extraction

of this latter urine yielded a small quantity of mercapturic acid. The experiment was repeated on a larger scale, and on the green-food diet a 3.5 per cent yield of mercapturic acid was obtained by chloroform extraction, while the method used by Abderhalden gave no trace of the substance. It would seem then that until the factors of diuresis and method of isolation are fully considered, the retardation of mercapturic-acid synthesis on a basic diet must be called in question.

In 1929 Nishimura (64) isolated mercapturic acid from the urine of rabbits in yields varying from 2.61 per cent to 9.4 per cent of the bromobenzene administered, and even obtained it from fasting animals. His method consisted in feeding an adequate diet, then fasting the animals for five or six experimental days during which 1 gm. of bromophenol was given daily. Results are reported as the combined yields for the experimental periods, and it is probable that more interesting and informative data would be furnished by the daily yields. Such data might show whether mercapturic acid was being formed at the expense of tissue cystine on the final days or all the mercapturic acid was formed on the first three days from so-called circulating protein.

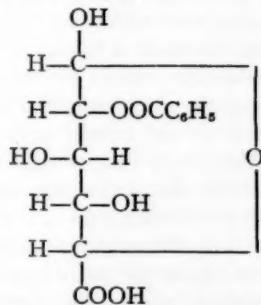
Abderhalden's experiments on fasting dogs (65, 66) show that the small amount of available endogenous cystine is immediately seized for such body needs as the formation of glutathione, taurine, and insulin. By limiting the preliminary fasting period to 1-2½ days and using smaller quantities of bromobenzene than previous investigators had done, he succeeded on a protein-free diet in isolating mercapturic acid even on the fifth or sixth experimental day. Occasional negative results were obtained; but it is probable that a more quantitative method would have detected the substance even in those cases. This work seems to prove conclusively that cystine is formed in tissue catabolism, and that this cystine can be utilized to detoxicate bromobenzene when there is no protein in the diet, provided the experimental conditions are not so extreme.

Recently, White & Lewis (58) have studied the metabolism of bromobenzene in dogs on cystine-deficient protein diets and cystine-rich protein diets, and found that mercapturic-acid formation, as judged by increase in neutral sulfur, parallels the cystine content of the proteins. More interesting is their simultaneous administration of bromobenzene and methionine to determine whether the latter could substitute for cystine in the formation of mercapturic acid.

They found that methionine was apparently as efficient as cystine in protecting the organism against excessive tissue catabolism and the toxic symptoms produced by bromobenzene on a low-cystine diet. There was also a similarity in the neutral sulfur figures given by cystine and by methionine. Unfortunately, it is difficult to interpret the sulfur figures of this experiment, because methionine alone caused an increase in neutral sulfur; and when this is added to the neutral-sulfur increase caused by bromobenzene alone, it accounts for most of the neutral-sulfur increase when bromobenzene and methionine are fed together. However, the results are indicative of a very close physiological relationship between cystine and methionine, and bring to our attention more forcibly the necessity of a good quantitative method for determining mercapturic acid, if such problems are to be settled definitely.

GLYCURONIC ACID

The study of glycuronic acid in mammalian physiology has been accelerated by the development of new quantitative methods. Csonka (67) has adapted the Benedict sugar method for the determination of glycuronic-acid monobenzoate, while Quick (68) has developed a method for the determination of borneol- and menthol-glycuronic acid which can also be applied to other conjugated acids that are soluble in ether. Methods for preparing glycuronic acid in pure crystalline form have been described by Ehrlich & Rehorst (69), Quick (70), Weinmann (71), and Link (72). Quick (73) has succeeded in isolating in crystalline form glycuronic acid monobenzoate, and has assigned to it the following structure:



The compound reduces Fehling's solution directly, shows mutarotation, and reacts with hydrocyanic acid without splitting off benzoic

acid; which is definite evidence that the aldehyde group is free. Recently the same author has isolated a diglycuronic-acid compound of *p*-hydroxybenzoic acid (74). In this unusual compound, one molecule is united in glycoside linkage, whereas the second one is in ester union. Evidence has also been obtained that salicylic acid undergoes a similar double conjugation. Miriam, Wolf & Sherwin (26) have isolated glycuronic-acid diphenylacetate.

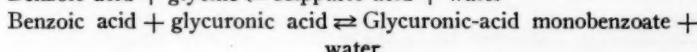
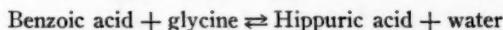
Metabolism.—In accordance with the older work, it has again been shown that the organism has a limited capacity to burn glycuronic acid, and that it has no power to alleviate insulin convulsions [Hurthle (75) and Quick (70)]. Glycuronic acid is fermented by various bacteria, notably by the coli-typhus group [Kay (76), Quick & Kahn (77)] and by *Aspergillus niger* [Hofmann (78)]. Conjugated glycuronic acids can be burned in the body. The fact that this is not generally realized has led to the fallacy of interpreting the output of conjugated glycuronic acid as a true measure of the animal's capacity to produce glycuronic acid. Quick (79) has shown that menthol and also menthol-glycuronic acid are nearly completely burned by the dog, and that in man glycuronic acid combined with benzoic acid and with *p*-hydroxybenzoic acid is burned, leaving the aromatic residue to become subsequently conjugated with glycine (74). Quick postulates that the conjugation of glycuronic acid presents perhaps the initial step in the oxidation of the particular compound undergoing conjugation, and that the conjugate appearing in the urine merely represents the fraction that has escaped complete oxidation.

The relation of glycuronic acid to carbohydrate metabolism has been studied by Quick. He has shown that a dog kept on a pure carbohydrate diet can synthesize as much as 5 gm. of glycuronic acid in 24 hours without any increase in nitrogen metabolism (80). He furthermore demonstrated that when a glycuronogenic drug was fed to a totally diabetic dog, the glycuronic acid was synthesized from the glycogenetic fraction of the protein molecule (81). Since the administration of insulin greatly increases the output of glycuronic acid (82), it seems probable that glucose can only be utilized for the synthesis of glycuronic acid through the action of insulin. Feeding of acetoacetic acid reduces the output of glycuronic acid. Quick postulates that the initial step in the catabolism of fatty acids may be a conjugation with glycuronic acid similar in type to the conjugation of benzoic and phenyl-acetic acids with glycuronic acid.

The effect of glycogenic amino acids upon the output of glycu-

ronic acid has also been studied (83). Two grams of menthol were fed to rabbits daily with varying quantities of the different amino acids, and the relation of nitrogen excretion to glycuronic-acid formation ascertained. Of the glycogenic acids—glycine, alanine, arginine, cystine, and glutamic acid—only the first three seemed to increase the output of glycuronic acid. Leucine, iso-leucine, cystine, and glutamic acid were doubtful in their influence. Valine, phenylalanine, and tryptophane had no effect, but tyrosine and histidine, both non-sugar formers, seemed to exert a decided influence in increasing the output of glycuronic acid.

The conjugation of benzoic acid with glycuronic acid has been studied in the pig by Csonka (67); in the dog by Brakefield & Schmidt (84) and Quick (85); in the rabbit by Griffith (86). In confirmation of Dakin's early work (87), Neuberg (88), Bignami (89), and Quick (90) have demonstrated that in man a small fraction of ingested benzoic acid is also excreted, combined with glycuronic acid. In attempting to determine why the conjugation of benzoic acid varies in different species, Quick (91) has applied the principles of the law of mass action to the reactions.



The basic factors in these reactions are: (a) the rate of the elimination of the end-products, hippuric acid and glycuronic-acid monobenzoate; (b) the concentration and supply of glycine and glycuronic acid; (c) the speeds of the reaction between benzoic acid and glycine and between benzoic acid and glycuronic acid. Substitutions introduced into the benzene ring of benzoic acid strikingly affect the conjugation, and substitution in the ortho position is most marked. A neutral group such as methyl exerts no demonstrable influence, an acidic group such as a halogen or the nitro group diminishes, while a basic group like the amino radical increases the output of conjugated glycuronic-acid compound (92).

Clinical studies on the output of glycuronic acid in liver have been made by Schmidt (93), Pozzi (94), and by Sauer (95). Nasarjanz (96) has reported that phosphorous poisoning lowers the output of menthol-glycuronic acid. Brakefield & Schmidt (84) found that in the dog the conjugation of benzoic acid with glycuronic acid was greatly diminished after ligation of the common duct. Quick &

Cooper (97) found no such diminution in experimental biliary obstruction, but noted a marked temporary decrease in the production of glycuronic acid after chloroform anesthesia. Eck-fistula dogs gave inconstant results. Kawanisi & Takee (98) reported that the administration of thyroid to rabbits increases the production of camphorol-glycuronic acid, while removal of the thyroid decreases the synthesis. Rygh & Rygh (99) claim that the synthesis of glycuronic acid is lost in severe scurvy. Quick (100), on the other hand, has found that the production of glycuronic acid appears to be normal even in the terminal stage of the disease.

The following table shows the fate of some of the derivatives of benzoic acid.

FATE OF SOME BENZOIC-ACID DERIVATIVES

Compound Fed	Free	Glycine	Glycuronic Acid
<i>o</i> -chlorobenzoic acid.....	Dog ^{1,2} , rabbit ²	Dog ²	Dog ²
<i>m</i> -chlorobenzoic acid.....	Dog, rabbit ¹	Dog ²	Dog ²
<i>p</i> -chlorobenzoic acid	Rabbit ¹	Dog ^{1,2}	Dog ²
<i>o</i> -bromobenzoic acid.....		Dog ^{1,2} , rabbit ¹	Dog ²
<i>m</i> -bromobenzoic acid.....	Rabbit ¹	Dog ^{1,2}	Dog ²
<i>p</i> -bromobenzoic acid.....		Dog ^{1,2} , rabbit ¹	Dog ²
<i>o</i> -aminobenzoic acid.....	Dog, rabbit, man ³	Dog ²
<i>m</i> -aminobenzoic acid	Dog, rabbit, man ³	Dog ²
<i>p</i> -aminobenzoic acid.....	Man, dog ³	Dog ²
<i>o</i> -iodobenzoic acid	Dog ³	Dog ^{2,3}	Dog ²
<i>m</i> -iodobenzoic acid.....	Rabbit ³	Dog partially ³
<i>p</i> -iodobenzoic acid		Dog ^{2,3} , rabbit ³	Dog (no red.) ²
<i>o</i> -, <i>m</i> -, and <i>p</i> -hydroxy- benzoic acid		Dog ²	Dog ²
<i>p</i> - and <i>m</i> -nitrobenzoic acid		Dog ²	Dog ²

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METHYLATION

Methylation, which is a common physiological reaction in plants, is seldom met in animal physiology. Tamura (19) injected a total of

9.5 gm. quinoline (in olive-oil solution) subcutaneously into dogs and isolated methyl-quinolinium hydroxide from the urine.

Novello, Harrow & Sherwin (101) fed imidazole, pyridine, quinoline, piperidine, and quinaldine to dogs and rabbits in the hope that a quantitative study of the methylation reaction would be possible. Of these substances, only pyridine was methylated in the organism of the dog, but no quantitative method could be developed for determining the methylated compound.

According to Kamei (102), pyridine, quinoline, and nicotinic acid, when administered to dogs having Eck's fistula, appear (to a much lesser degree than in normal dogs) as methylated derivatives in the urine. He concludes that the liver is important in methylation reactions. Stuber (103) claims that guanidine-acetic acid, injected intravenously into rabbits, is converted into creatine by methylation. The removal of the thyroid inhibits this reaction, while feeding of thyroid or inorganic iodides restores the last function. He believes that the thyroid gland is closely associated with the mechanism of methylation.

ETHEREAL SULFATE

Hydroxy compounds, either aliphatic or aromatic, are usually detoxicated by conjugation with sulfuric acid and excreted as ethereal sulfate; or in case the supply of sulfuric acid is insufficient, glycuronic acid may take the place of the sulfate. There is still a question whether sulfuric-acid conjugation or glycuronic-acid conjugation is first employed by the animal body. It probably differs in different species of animals and also varies with different compounds. The more recent investigations have dealt with the origin of the ethereal sulfates.

Shipley, Muldoon & Sherwin (46) maintained a pig on a carbohydrate diet and fed the animal bromobenzene, phenol, and *p*-chlorophenol after the animal was reduced to a condition of endogenous nitrogen catabolism. They noted a distinct rise in the output of ethereal sulfate. The ethereal-sulfate fraction was augmented by simultaneous feeding of inorganic sulfate. However, the feeding of cystine together with these compounds and in the presence of phenol increased the ethereal sulfate, but did not increase the ethereal-sulfate output when cystine was fed with the other two substances; it rather caused a decrease in this type of sulfate when bromobenzene was fed.

They conclude that in the absence of exogenous cystine, the phenolic compounds are detoxicated with the sulfate radical obtained from tissue destruction and readily excreted as ethereal sulfate. They believe that in the presence of exogenous cystine, mercapturic acid is formed and excreted as such, or oxidized to an ethereal sulfate. Similar results were obtained on dogs.

Hele (56), on the other hand, believes that the dog can utilize sodium sulfate in the formation of ethereal sulfate and concludes that the dog is able to utilize inorganic sulfates from an exogenous source. He also believes that the sulfate ion combines directly with phenolic compounds.

Coombs & Hele (54) found that the pig could not synthesize ethereal sulfate as readily as the dog. They noted an increase in reducing substance in the urine and believed the substance due to glycuronic acid. They do not believe that mercapturic acid is utilized to form ethereal sulfate.

White & Lewis (58) found after the feeding of bromobenzene on a low-cystine diet that there is a rise in ethereal sulfate together with neutral sulfur; from which they conclude that on a low-cystine diet less cystine is available for mercapturic-acid formation, and consequently more ethereal sulfate is formed. They noted a corresponding decrease in inorganic sulfate with an increase in ethereal sulfates. They conclude that after feeding bromobenzene with either cystine or methionine, the extra cystine in the diet is used for the formation of mercapturic acid; and when the cystine sulfur in the diet is lowered, some of the bromobenzene is oxidized to bromophenol, which in turn is conjugated with sulfuric acid, or with sulfate, increasing the ethereal-sulfate fraction.

Stekol & Cerecedo (104) have shown that, when isobarbituric acid is fed to dogs, 22 to 30 per cent of the substance appears in the urine as ethereal sulfate. The ethereal sulfate was isolated from the urine and identified as the xanthydrol derivative. This work was confirmed by Lawrie & Pirie (105).

Aloxan when fed to dogs showed a distinct drop in the output of inorganic sulfate in the urine, and it is assumed that aloxan may be partially excreted in the bile, possibly as an ethereal sulfate (106). Stefanovich (107) noted that parathyroidectomized or thyroidectomized dogs with gastric fistulae showed an increase in ethereal-sulfate output after the administration of hydroxyquinone, while complete thyroparathyroidectomy caused a drop in ethereal sulfate.

ACETYLATION

Acetylation thus far has been observed only in the case of amino compounds. Acetylation had previously been reported by Ellinger & Hensel (108) in the case of *m*- and *p*-amino-benzoic acid by the rabbit. Muenzen, Cerecedo & Sherwin (109) reported the acetylation of *m*- and *p*-amino-benzoic acids in the human being. Acetylation of α -amino-phenylacetic acid has also been noted in the case of rabbits (110). The chicken appears to be able to acetylate *m*-amino-benzoic acid (111). These appear to be the first acetylation reactions recorded where human beings and fowls were used as subjects.

It is interesting to note that, while the amino-benzoic acids and amino-phenylacetic acids (particularly the para compounds), when fed to human beings or rabbits, are acetylated, the same compound in the dog is detoxicated by a conjugation with glycine, forming an amino-hippuric acid. Dogs, on the other hand, seem to acetylate an amino group in the side chain such as α -amino-phenylacetic acid, forming α -acetyl-amino-phenylacetic acid.

Knoop & Blanco (112) find that when *d,l*-acetyl derivatives are administered, the *l*-form predominates in the urine. Hydroxy and keto acids are also eliminated, indicating a decomposition similar to the non-acetylated amino acids. The place of acetylation is undoubtedly in the liver.

The source of acetic acid has been studied by Harrow, Power & Sherwin (113). These investigators fed *p*-amino-benzoic acid to rabbits until the output of acetyl-amino benzoic acid was determined with the given dosage. They then fed the experimental animals various substances and determined the increase in the quantity of amino-benzoic acid acetylated. The following numbers represent the percentage of increase: tyrosine, 188; ethyl ester of acetic acid, 176; sodium acetate, 158; β -hydroxybutyric acid, 133; pyruvic acid, 126; glyceric aldehyde, 118; histidine, 102; glycerol, 93; acetic aldehyde, 81; olive oil, 52; serine, 49; nor-leucine, 42; fructose, 40; glycine, 28; alanine, 27; lactic acid, 22; glucose, 16; tryptophane, 15; and iso-leucine, 12.

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DEPARTMENT OF CHEMISTRY
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PLANT PIGMENTS*

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I. THE CAROTENOIDS

During the year covered by this review no new naturally occurring carotenoids have been isolated in the pure state. Spectroscopic data, however, indicate that perhaps another, still unknown carotenoid exists in carrots besides β -carotene and α -carotene (1, 2), and that the fruits of *Gonocarium obovatum* and *G. pyriforme* contain possibly a new representative of this class of pigments (3).

Various authors have discovered new sources of the known carotenoids. Winterstein & Ehrenberg isolated carotene, lycopin, and xanthophyll from the fruits of the lily-of-the-valley (*Convallaria majalis*). The same authors found lycopin in the fruits of bryony (*Bryonia dioica*), and they isolated physalene from the sandthorn berry (*Hippophae rhamnoides*), from *Solanum Hendersonii*, *Lycium barbarum*, and from asparagus berries (*Asparagus officinalis*). Spectroscopic analysis showed that lycopin is probably also contained in the fruits of *Erythroxylon coca* (3). Lycopin, zeaxanthin, and presumably some carotene occur in the fruit of the red kaki (*Diospyros kaki*) (4). Karrer & Notthafft (5) analyzed numerous yellow blossoms and found various oxygen-containing carotenoids: xanthophyll in the blossoms of *Caltha palustris L.*, *Trollius europaeus*, and *Ranunculus arvensis L.*; xanthophyll in addition to violaxanthin in *Tragopogon pratensis L.* (subspecies *orientalis*); violaxanthin in *Tragopogon pratensis*, laburnum, and *Sinapis officinalis*; taraxanthin in *Tussilago farfara* (6); and zeaxanthin in the flowers of *Senecio doronicum L.* All these pigments are present in the esterified form. These are not the only ones present, but they are the prevailing carotenoid pigments occurring in the flowers mentioned.

The existence of lycopin in flowers had previously not been demonstrated, but Zechmeister & Cholnoky (7) have now found it in the dark yellowish-red flowers of *Calendula officinalis*, and Karrer & Notthafft (5) have found it in the flowers of *Dimorphotheca aurantiaca* and in violet-blue vetches. The latter report that carotenoids seem also to occur quite frequently in flowers of colors other than

* Received December 28, 1932.

yellow. Crocetin is present in the flowers of mullen (*Flores verbasci*), [Schmid & Kotter (8)].

Several workers report the occurrence of carotenoids, especially carotene, in the animal organism. For example, Baily & Netter have demonstrated the presence of carotene in the suprarenal gland (9), Weissberger & Bach in the skin of gold fish (10), and H. von Euler, Gard & Hellström in the petroleum-ether extract of the spawn of various fishes (*Solea vulgaris*, *Gadus calarias*, *Hippoglossus hippoglossus*, *Lota vulgaris*, *Esox lucius*). In the case of *Hippoglossus*, zeaxanthin and xanthophyll were also found, and in *Lota vulgaris* carotene and xanthophyll (11). According to Kuhn and Brockmann (12), the human placenta contains carotene and xanthophyll, while the corpora lutea of cows contain β -carotene.

A colored species of bacteria, *Sarcina lutea*, seems to contain a new pigment of the carotene series, namely sarcinin. Its existence was demonstrated spectroscopically. In addition to sarcinin, a pigment related to xanthophyll seems to be present (13).

A quantitative separation of different carotenoids is very often required because of the frequency with which they occur simultaneously in plants and animals. Employing the old methods chiefly worked out by Willstätter, that is by distributing the pigments between petroleum ether and aqueous methanol it is in most cases easy to separate the carotenoids of the hydrocarbon type from the compounds containing oxygen. For the latter type Karrer has proposed the group name, phytoxanthins (5). The carotenoids of the hydrocarbon type dissolve in the petroleum-ether layer together with the esters of the phytoxanthins, while the free phytoxanthins are retained by the methanol.

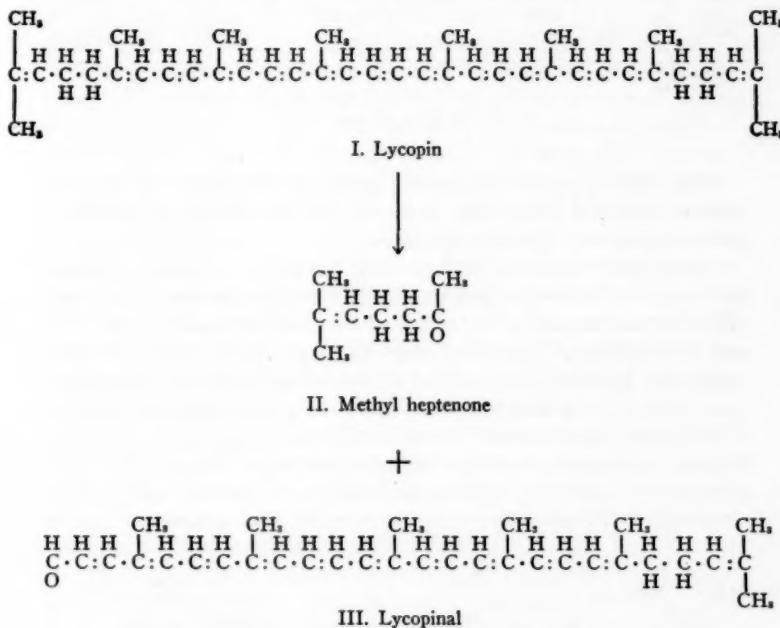
The separation of the individual hydrocarbons and of mixtures of phytoxanthins can be accomplished by Tswett's method of chromatographic analysis, which lately has been so perfected by Kuhn & Brockmann that the separation of complex mixtures is now possible (12). The authors use fiber clay as an adsorbent for carotenoid-hydrocarbon mixtures and calcium carbonate for phytoxanthins.

In a similar way Karrer & Schöpp have worked out methods for the separation of vitamin A from carotene or phytoxanthins (14).

An attempt to improve the method of isolating carotene from green leaves is reported by Homes & Leicester (15), and also by J. H. C. Smith (21). The carotene content of alfalfa, dried and fresh spinach, yellow corn gluten, canned carrots, leaves of black figs, sugar beets, cauliflower, and the sunflower is also given. An extensive

comparison of the Carr-Price reaction of carotenoids is the subject of a publication by B. von Euler & Karrer (16).

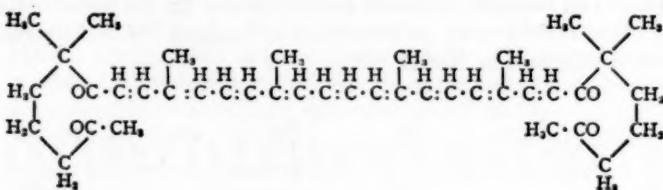
1. *Polyene hydrocarbons*.—Kuhn & Grundmann have succeeded in bringing about a partial oxidation of lycopin (I) by means of chromic acid (17). The resultant products were methyl heptenone (II), and an aldehyde, lycopinal, which probably has the constitution III. This reaction may be considered as support for the lycopin formula suggested by Karrer (18).



Lycopinal melts at 147°. The absorption bands of maximum intensity are at 525.5, 490.5, and 455.5 m μ in petroleum ether; at 569, 528.5, and 493.5 m μ in carbon bisulphide.

In the study of α -carotene Karrer and collaborators found that the last mother liquors from the preparation of carotene from carrots contain a carotene which shows the same absorption as α -carotene but has a much smaller specific rotation. It is therefore possible that some levo form occurs in the carrot besides the dextro α -carotene (2).

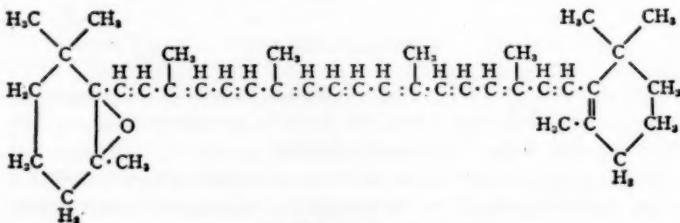
β -carotene on mild oxidation with chromic acid yields an oxy-carotene ($C_{40}H_{58}O_3$) which still has good growth-promoting activity, and a tetraketone, β -carotenone (IV) formed by the splitting of the two carotene carbon-rings [Kuhn & Brockmann (19)].



IV. β -carotenone

This ketone shows absorption bands in the region of higher wave-lengths than β -carotene. It forms a dioxime and has no growth-promoting activity even in large doses.

An oxygen derivative of β -carotene having the oxygen bound in the form of an oxide was prepared by H. von Euler, Karrer & Walker (20). It was formed by the action of 1 mol of perbenzoic acid on 1 mol of β -carotene. The yield was 10-15 per cent. The resultant compound, called carotene oxide (V), crystallizes well, has a melting-point of 161°, and its absorption bands are in the region of shorter wave-lengths than those of β -carotene. Carotene oxide on distribution between petroleum ether and aqueous methanol behaves like the polyene hydro-carbons, in the sense that it passes into the petroleum-ether layer. The probable structural formula is as follows:



V. Carotene oxide

Carotene oxide shows good vitamin-A activity.

In order to further clarify the constitution of α -carotene, experiments on the ozonization of the pure compound have been performed. Neither geronic acid nor isogeronic acid was formed. This behavior of α -carotene proves that the double bonds in the carbon rings cannot have the same position as in β -ionone or α -ionone (4).

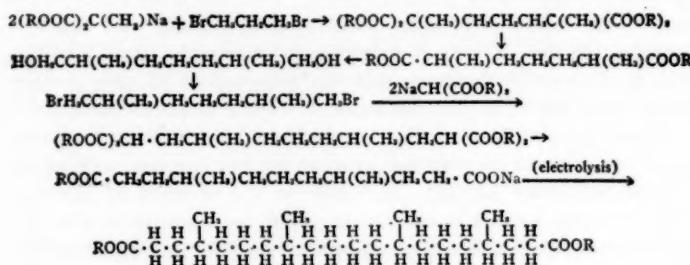
Some new work on the catalytic hydrogenation of lycopin and carotene is reported by J. H. C. Smith (21). He finds that lycopin takes up 13 mols of hydrogen, which is in agreement with Karrer's findings. On the other hand, carotene added on only 10 mols of hydrogen. This result disagrees with the observations of several other authors who found by hydrogenation 11 double bonds in carotene.

When β -carotene tri-iodide or carotene tetra-iodide is decomposed with sodium thiosulphate, one can recover carotene with all its original properties (2, 22). If, however, the solution of carotene tetra-iodide in acetone is allowed to stand for some time, and one tries to remove the iodine later on by shaking with a thiosulphate solution, an isomer of carotene, known as isocarotene ($C_{40}H_{56}$), is obtained [Kuhn & Lederer (23)]. The melting-point of this compound is higher than that of carotene, 192° to 193° corr. (2), its absorption bands are in the region of higher wave-lengths (543, 504, 472 μ in CS_2), and it has no growth-promoting activity. On hydrogenation it takes up 12 mols of hydrogen [Karrer, Schöpp & Morf (2)], and on ozonization it does not form geronic acid. On oxidation with potassium permanganate it yields 1,1-dimethyl glutaric acid, and 1,1-dimethyl succinic acid, as well as traces of a dicarboxylic acid with a higher melting-point (possibly succinic acid). The authors believe that in the case of isocarotene one of the two carbon rings of β -carotene is open while the second ring remains closed, with a shift, however, in the position of the double bond.

Small amounts of isocarotene are sometimes formed in the preparation of carotene oxide from β -carotene and perbenzoic acid (20).

2. *Polyene alcohols*.—Karrer, Morf, Krauss & Zubrys (4) have converted zeaxanthin into the corresponding hydrocarbon by substituting the two hydroxyl groups of completely hydrogenated zeaxanthin first by bromine and then by hydrogen. The hydrocarbon so obtained was optically inactive. Another hydrocarbon, prepared in an analogous manner from perhydro-xanthophyll, proved to be dextro-rotatory. It is therefore likely that zeaxanthin is derived from optically inactive β -carotene, while xanthophyll originates from the dextro-rotatory α -carotene.

3. *Polyene carboxylic acids*.—The constitution of bixin (VII) has been definitely established by the synthesis of perhydro-norbixin and the diethyl ester of perhydro-norbixin (VI). The synthesis was carried out by Karrer, Benz, Morf, Raudnitz, Stoll & Takahashi (24). The different stages of the procedure were as follows:



VI. Perhydro-bixin ester

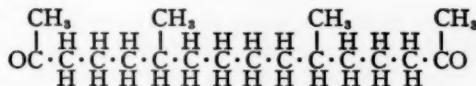
The crystallized diamide and the crystallized di-(2,4,6-tribrom)-anilid of the synthetic perhydro-bixin had the same melting-point as the corresponding derivatives from native bixin. Mixtures of the synthesized and natural derivatives showed no depression of the melting-point.

This proves that bixin has a symmetrical structure like lycopin and carotene as far as the arrangement of the methyl groups is concerned.



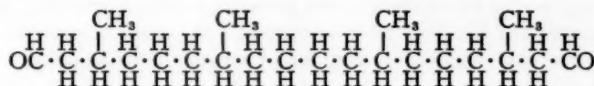
VII. Bixin

The same is true for the saffron pigment, crocetin, because perhydrocrocetin was transformed by Karrer and collaborators (24) into 6,11-dimethyl-hexadecane-dione (2, 15) (formula VIII), by a series of reactions consisting of bromination, treatment with potassium hydroxide, methylmagnesium halide, and lead tetra-acetate.



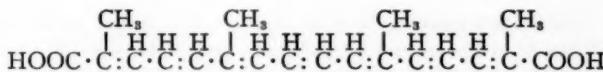
VIII. 6,11-dimethyl-hexadecane-dione

Under similar conditions of reaction a dialdehyde (IX) was formed from perhydro norbixin.



IX

The older crocetin formula is no longer valid and has to be replaced by the following (X) :



X. Crocetin

According to Kuhn it is possible that crocetin and bixin are oxidation products of the carotenoids with 40 C-atoms, the centers of which remained unchanged, while the ends were removed by oxidation. The arrangement of the 20 C-atoms of crocetin and the 24 C-atoms in norbixin is the same as in lycopin, carotene, and its oxygen derivatives (19).

If bixin and the stereo-isomeric isobixin (trans- form) are reduced to the dihydro stage, one obtains, as expected from theory, identical products [Kuhn & Winterstein (25)]. On reduction of these two compounds a shifting of the double bonds takes place, whereby the cis-trans- isomerism is eliminated. Dihydro-crocetin and dihydro-bixin are reconverted comparatively easily into crocetin and bixin by atmospheric oxygen in the presence of strongly basic or tertiary amines (26). Kuhn believes it very likely that the dihydro com-

pounds undergo enolization under the influence of the active bases and that the enols, by shifting their conjugated double bonds, undergo dehydrogenation. This assumption is supported by the fact that the dimethyl ester of dihydro-crocin, dissolved in pyridine with a little sodium hydroxide, gives a deep blue solution containing possibly the sodium salt of the enol form of dihydro-crocin-dimethyl-ester. Dihydro-methyl-bixin under the same conditions yields a deep emerald-green solution (27).

A great number of publications relative to the activity, distribution, and testing of vitamin A, the growth-promoting vitamin, which was recognized last year as a carotenoid (28), have appeared. The problem of the chemical constitution of vitamin A is the subject of a report by Heilbron and collaborators (29). These authors confirm essentially Karrer's observations. Heilbron, Morton & Webster (30) subjected vitamin A to dehydrogenation with selenium and found the resultant compound to be 1,6 dimethyl naphthalene, which is in accordance with the formula already proposed (28). This confirms the arrangement of the first 14 carbon atoms.¹

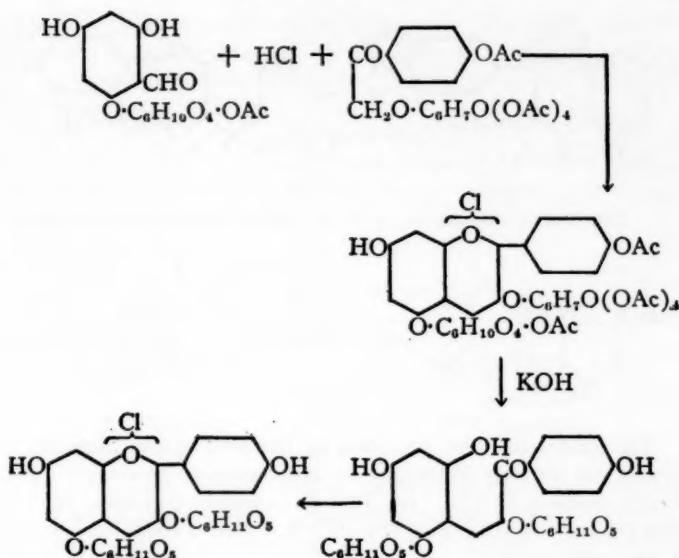
II. PYRANE AND QUINONE PIGMENTS

1. *The anthocyanins.*—A new yellow anthocyanin called "gesnerin" has been discovered by Robinson and Robinson (31) in the orange-red flowers of *Gesnera fulgens* (or *Gesnera cardinalis*). It is a 5-glucoside of 4',5,7-trihydroxy-flavilium chloride, although the nature of the sugar is still unknown. On hydrolysis it yields apigenin chloride which had been synthesized previously by Pratt & R. Robinson (32). Gesnerin is consequently the flavilium compound related to the flavone pigment apigenin, and represents the first anthocyanin pigment found in nature which has no hydroxyl group attached to carbon atom 3.

During the last year great progress has been made in elucidating the constitution of the diglucoside anthocyanins. The assumption² that the two sugar radicals in pelargonin, malvin, cyanin, and several other anthocyanidin diglucosides are separately attached to the hydroxyls 3 and 5 proves to be correct. Several of these compounds have been synthesized by R. Robinson and Todd (33). The synthesis of pelargonin (XI), for example, takes place in the following stages:

¹ For formula, cf. Karrer, P., *Ann. Rev. Biochem.*, **1**, 557 (1932).

² Cf. *Ann. Rev. Biochem.*, **1**, 564 (1932).

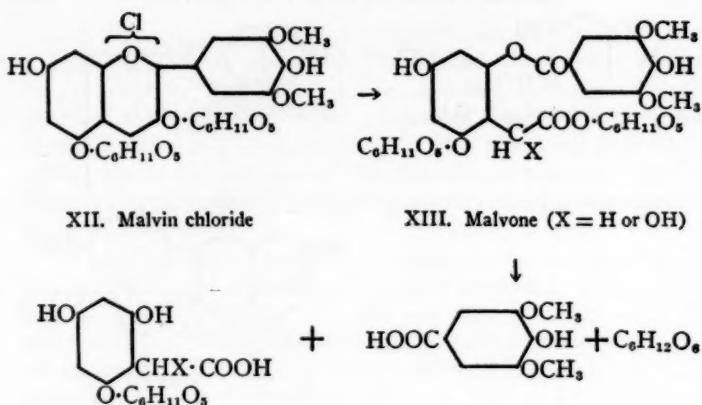


XI. Pelargonin

The syntheses of paeonin, cyanin, malvin, and hirsutin were carried out in a similar manner.

Another proof for the independence of the two sugar radicals in the anthocyanins mentioned above was furnished by Karrer & deMeuron (34). They showed that malvone, which is formed by the oxidation of malvin (XII) with H_2O_2 , has the structure represented by formula XIII. This is established by the fact that malvone on mild alkaline saponification, or by the action of phenyl-hydrazine, loses easily the one sugar radical which was originally attached to hydroxyl group 3 of malvin, while the second sugar radical which is attached by a glucoside linkage can only be split off by acid hydrolysis.

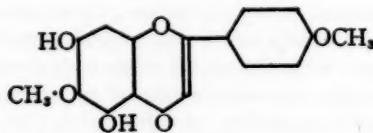
The diglucosides monardin, paeonin, and cyanin after oxidation with hydrogen peroxide react in the same way as malvin, if subjected to alkaline hydrolysis or to the action of phenyl-hydrazine; one sugar radical is split off, while the other remains attached to the phloroglucin portion of the molecule. The former occupies position 3 in the pigment molecule.



The shades of color exhibited by the anthocyanin pigments in flowers are strongly influenced by accompanying substances (35). Tannin, flavones, flavonols, etc., may act as such co-pigments. In most cases the color shifts toward blue. Little is known about the isolation of these co-pigments.

Observations on the variation in the colors of flowers in inheritance tests were reported by Onslow (36). The recessive forms frequently contain the pigment with less oxygen. Plants containing delphinidin can transmute to plants with cyanidin or pelargonidin. Occasionally, however, the reverse is observed.

2. *Flavones, xanthones.*—From the flowers of toad flax (*Linaria vulgaris*), Schmid & Rumpel (37) isolated a flavone pigment having two sugar groups, the one a hexose, the other a methyl pentose. The aglucone seems to have the structure given by formula XIV.

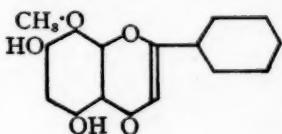


XIV

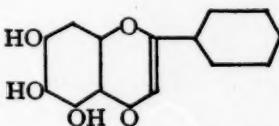
The compound in question is therefore the 4',6-dimethyl-ether of scutellarein, which is obtained on decomposition with hydriodic

acid. Alkaline decomposition gives anisic acid. The diacetyl derivative proves to be identical with the diacetate of a compound of the structure described above, which was, indeed, synthesized earlier by Wessely & Moser (38).

Hattori (39) has investigated anew wogonin (XV) and baicalein (XVI). He finds that the earlier conclusions of Shibata & Hattori (40) concerning the constitution of the two pigments are wrong. Actually their structures appear to be as follows:



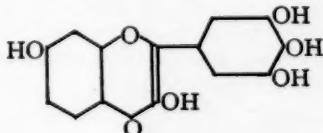
XV. Wogonin



XVI. Baicalein

The trihydroxy flavone resulting from demethylation of wogonin proved to be identical with a product obtained by dealkylation of synthetic 5-ethoxy-7,8-dimethoxy flavone. The constitution of baicalein follows from that of its trimethyl ether, which on prolonged boiling with dilute KOH yields 3,4,5-trimethoxy phenol (antiarol), besides acetophenone and benzoic acid.

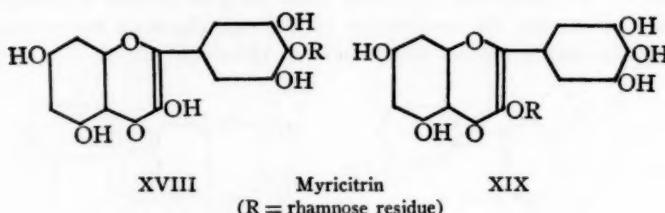
Brass & Kranz have investigated the yellow pigment of the wood from *Gleditschia monosperma* (41). It proves to be identical with the one isolated by Schmid & Pietsch from *Robinia pseudoacacia* (42). It has the empirical formula $C_{15}H_{10}O_7$, and forms a penta-acetyl compound (m.p. 224°) and a pentamethoxy compound (m.p. 148°). The decomposition of the latter with a methyl alcohol solution of potassium hydroxide gave the trimethyl ether of tannic acid and fisetol dimethyl ether (2-hydroxy-4, ω -dimethoxy-acetophenone). It follows, therefore, that the pigment of acacia wood is 3,7,3',4',5'-penta-hydroxy flavone (5'-oxyfisetin) (XVII).



XVII. 5'-oxyfisetin

This finding has been confirmed by Schmid & Tadros (43).

The rhamnoside, myricitrin, which was given formula XVIII by Perkin (44), receives confirmation through the work of Hattori & Hayashi (45) who propose formula XIX.



The sugar residue is in position 3 because the pentamethyl ether of myricitrin yields on acid hydrolysis 5,7,3',4',5'-pentamethoxy flavonol, a product identical with a synthetic preparation.

Robinin ($C_{43}H_{40}O_{19}$), a caemphesterol glucoside, contains a tri-saccharide as its sugar component. It consists of 1 mol of galactose and 2 mols of rhamnose (46).

Hattori has completed and summarized his study on the absorption of light by the oxyflavones (47). He found regular relations between absorption and constitution which prove to be helpful in the explanation of structure in the flavone group. The original should be consulted for many important conclusions.

Mangostin is believed to be a xanthone derivative of complex structure. Murakami (48) has worked on its constitution and suggests a formula which, however, seems to be rather uncertain.

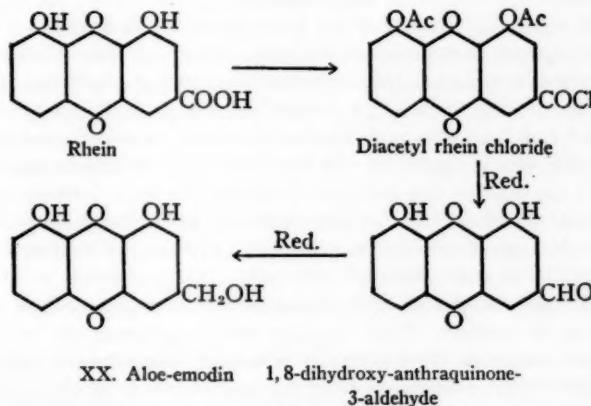
3. *Pigments of other groups.*—Gossypol, the pigment of cotton seeds, has been the subject of a new investigation by Karrer & Tobler (49). The authors confirmed the empirical formula $C_{30}H_{30}O_8$ and identified in the substance two neighboring carbonyl groups by proving that gossypol and *o*-phenylenediamine react to form a derivative of quinoxaline. Gossypol is therefore either an α -diketone or an *o*-quinone. Ozonization gave gossypolic acid ($C_{12}H_{14}O_4$)_x in small quantities. The acid was identified as an aromatic hydroxy acid by its intense purple color in the ferric chloride test. With diazomethane it forms an ether acid.

Monascin ($C_{24}H_{30}O_6$), a beautifully crystalline yellow pigment,

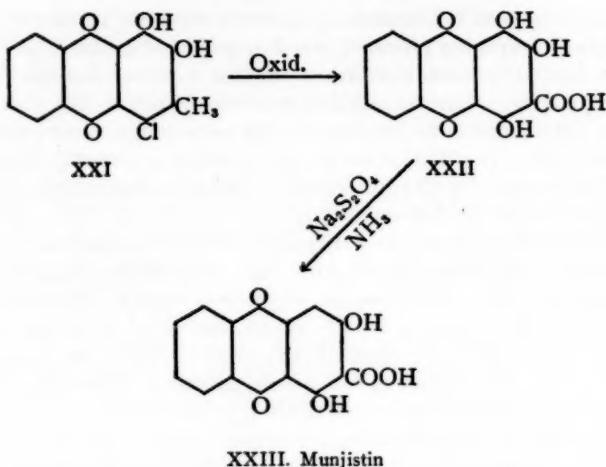
has been isolated by Salomon & Karrer (50) from the red fungus *Monascus purpureus* (*Wentii*), which is cultivated on rice (red rice). It is formed by such cultures in addition to a red substance and seems, mainly, to have an aliphatic structure (m.p. 135–140°). Nishikawa (51) found in the mycelium of the same fungus a red pigment, monascorubin ($C_{22}H_{24}O_5$) (m.p. 136°), which is probably different from monascin. Its dihydro derivative forms reddish-yellow prisms. Both substances are levorotatory.

The residues from the extraction of ergot contain two yellow pigments, ergoflavin ($C_{15}H_{14}O_7$) and ergochrysin ($C_{21}H_{28}O_{12}$) [Bergmann (52)]. Ergoflavone which was already discovered by Freeborn (53) contains 5 OH- groups and is of a lactone type; ergochrysin is probably identical with secalonic acid, discovered by Kraft (54). The latter, on fusing with potassium hydroxide, yields oxalic acid, 1,3,5-cresotinic acid ($COOH : CH_3 : OH = 1 : 3 : 5$), resorcin and 2,4,2',4'-tetrahydroxy biphenyl.

Aloe-emodin (XX) and munjistin (XXIII), two hydroxy anthraquinone pigments occurring in nature, have been synthesized by Mitter and collaborators (55). The aloe-emodin synthesis proceeds as follows:



Munjistin was prepared from chlor-dihydroxy-methyl anthraquinone (XXI), through the carboxylic acid (XXII) (56):



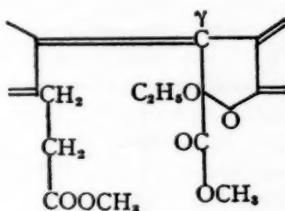
XXIII. Munjistin

III. CHLOROPHYLL PIGMENTS

1. *Chlorophyll-a*.—Phaeophorbid-*a*, which can be prepared by the careful splitting-off of magnesium and phytol from chlorophyll-*a*, is best suited for analysis of the latter because it is considered to be the undamaged chromophore-component of the pigment. The empirical formula of phaeophorbid-*a* (as the mono methyl ester) was at first considered to be $\text{C}_{35}\text{H}_{36}\text{O}_5\text{N}_4$ (Willstätter and Stoll, 1913), while Fischer and Conant in their studies of recent years proposed a formula with one oxygen atom (H_2O) more. Stoll & Wiedemann were able on the basis of new analyses to confirm the older formula. They explained the deviation of Fischer's results by the hygroscopicity of many chlorophyll derivatives (57). This view has recently been accepted by Fischer (58).

Phaeophorbid-*a* and its degradation products change their composition in solution. They undergo dehydrogenation or, in other words, allomerize. This behavior is a great obstacle and makes it difficult to draw conclusions from the degradation products about the constitution of phaeophorbid-*a*. The allomerized chlorophylls are characterized by the loss of "phase" which consists of a temporary shift of the chlorophyll-*a* derivatives from green to yellow, and of the -*b* derivatives from green to red, under the influence of strong

alkali. Allomerization can also be effected arbitrarily. Subsequent degradation with hydrogen iodide leads to different phaeoporphyrins, depending upon the conditions. The dehydrogenation of phaeophorbid-*a* by means of oxygen or iodine gives phaeoporphyrin-*a*₅ and -*a*₇. Fischer recently studied phaeoporphyrin-*a*₆, which is prepared from chlorophyllid-*a* by dehydrogenation with quinone in absolute alcohol (59). Phaeoporphyrin-*a*₆ cannot be transformed into the known members of this series, but yields on oxidation with fuming sulphuric acid an isophaeoporphyrin-*a*₇, and on treatment with alkalies an iso-chloroporphyrin-*a*₆. Fischer suggests formula XXIV for part of phaeoporphyrin-*a*₆.



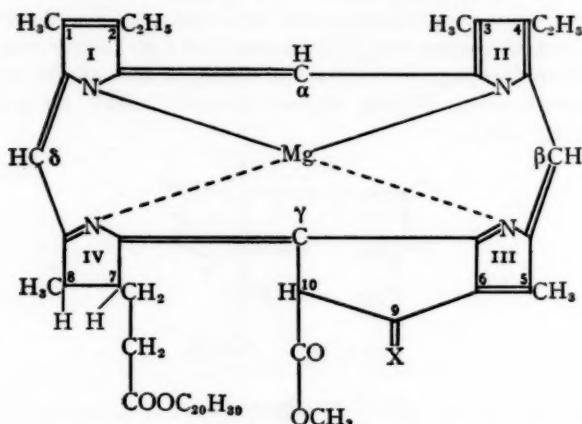
XXIV. Phaeoporphyrin-*a*₆

By avoiding allomerization and by careful degradation with the aid of iodine-free hydriodic acid, Stoll & Wiedemann prepared from phaeophorbid-*a* a protophaeoporphyrin-*a* which is well characterized by the positive-phase test. The new porphyrin changes easily to phaeoporphyrin-*a*₅ on dehydrogenation (60). The results of the degradation and analysis form the basis for formula XXV (p. 412)⁸ for chlorophyll-*a*.

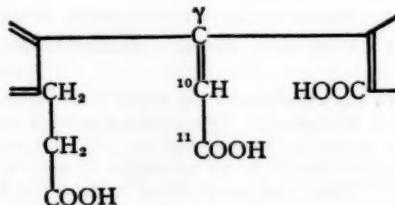
Stoll & Wiedemann's evidence for an alcohol group on C₉ is based, primarily, upon the preparation of a benzoyl ester of phaeophorbid-*a*. Fischer explains this ester formation, however, by a possible enolization of his keto group. Allomerization, according to

⁸ To explain the pigment character the double bonds are written in regular conjugation (Stoll & Wiedemann). This permits a multiple tautomerism which could be used as an explanation of isomerisations (61). The newest formulation given by Fischer is characterized by the assumption of methylene groups as side chains in positions 1, 3, and 5 and imino groups in the pyrrol rings III and IV. This assumption is supported by the fact that active hydrogen can be detected in the Zerewitinoff test (62).

Fischer, leads to a double bond between C₁₀ and C₇, while Stoll & Wiedemann assume that a keto group is formed at C₉, which accords with Fischer's assumption for native chlorophyll. The existence of the keto group in the allomerized chlorophyll derivatives is confirmed by the preparation of the oximes.

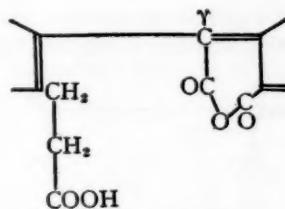
XXV. Chlorophyll-*a* $X = O$ (Fischer) $X = HOH$ (Stoll & Wiedemann)

Phaeophytin-*a* and phaeophorbid-*a* are converted by the action of alkalies into various chlorins, depending upon the experimental conditions. On rapid saponification in the heat, chlorin-*e* (formula XXVI, according to Fischer) is obtained (58).

XXVI. Chlorin-*e*

The assumption of a double bond between C_γ and C₁₀, which is supposed to add on water reversibly, explains the varying analytical results observed in the determination of oxygen. The free acid of chlorin-*e*, for example, was found to have seven oxygen atoms while the trimethyl ester had only six. The discrepancies in the results of the degradation with hydroiodic acid may also be interpreted in this way. Degradation of the free chlorin-*e* and of its dimethyl ester led to chloroporphyrin-*e*₅ (γ -formylrhodoporphyrin), while the trimethyl ester yielded chloroporphyrin-*e*₆ (rhodoporphyrin- γ -acetic acid). Chlorins (and also the phorbids) seem to conform with the formulae of dihydroporphyrin. This assumption is in agreement with Conant's observation of the structure of the spectra at the temperature of liquid air, whereby a parallelism between porphyrin-benzene on the one hand and chlorin-dihydro-benzene on the other was found (63).

Phaeophorbid-*a* on treatment in the cold with ethyl alcoholic or propyl alcoholic potassium hydroxide leads to unstable chlorins which, in solution, gradually change to purpurins. Applying this mode of saponification, Steele found volumetrically that 1 mol of O₂ was taken up (64). The purpurins, as a consequence, have to be considered as oxidation products of chlorophyll. However, they still contain the porphin system because Fischer could prepare ferric salts having properties similar to the ferric salts of the porphyrins (65). Up to the present 2 purpurins have been isolated—phaeopurpurin-7, which according to Conant has the side chain grouping of a rhodoporphyrin- γ -glyoxylic acid, and purpurin-18, which Fischer believes to have the grouping of rhodoporphyrin- γ -carboxylic anhydride (XXVII). This assumption is supported by the fact that purpurin-18 is easily transformed into the porphyrin in question under various conditions (66).



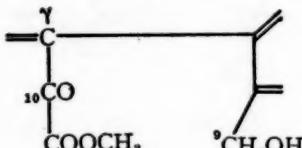
XXVII. Purpurin-18

Before crystallization takes place, purpurin-18 seems to be in solution as a tri-carboxylic acid, because it is possible to prepare a trimethyl ester by means of diazo-methane. Crystallized purpurin-18, on the other hand, forms a mono-methyl ester.

The transformation of chlorins into purpurins with the aid of hydrolyzing agents is reversible. From purpurin-18 one obtains in this way, chlorin-*p*₆, which also corresponds to the rhodoporphyrin- γ -carboxylic acid, as confirmed by synthesis and degradation (66).

Purpurin-18 was isolated by Fischer from the excrement of the silkworm, where it also occurs in small quantity with phyllobombycin. Spectroscopic data indicate that phyllobombycin is also a purpurin, and its analysis and degradation point to a close relation to chlorin-*e* (XXVI), (67). By the isolation of phylloerythrin and probophorbids from the excrement of sheep, Fischer was able to collect more data concerning the catabolism of chlorophyll (67). The components *a*, *c*, and *d* of the probophorbids are similar to desoxy pyrrophaeophorbid-*a*. The latter can be prepared by decarboxylation of phaeophorbid-*a* with sodium carbonate in pyridine solution and from chlorin-*e*-trimethyl ester by closing the carbon ring and decarboxylating with the same reagent (68). All these phorbids may be derived from the formula of phylloerythrin (XXIX).

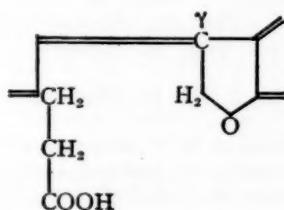
2. *Chlorophyll-b*.—Chlorophyll-*b* differs from the compound -*a* by one additional oxygen atom, the ketone nature of which is established [Conant (69), Warburg (70), Fischer (71), Stoll & Wiedemann (72)]. The setting-up of a structural formula meets with much difficulty so that no satisfactory conclusion has been reached. Conant believes that the constitutional difference between the two chlorophylls is chiefly due to a keto group in chlorophyll-*b* instead of a methin group on carbon β . Fischer considers it far more likely that this difference is centered in the propionic-acid radical. Stoll & Wiedemann, finally, are of the opinion that chlorophyll-*b* has no iso-cyclic ring and that the part of the molecule made up by C-atoms 9 and 10 has the following structure (formula XXVIII):



XXVIII

Stoll & Wiedemann assume a primary alcohol group on C₉ which is supported by the reversibility of the allomerization of chlorophyll-*b*, brought about by reducing agents. Fischer and Conant instead have a lactone bond between C₉ and C₁₀ or C₁₁ (see 69, 71).

3. *Synthesis of chlorophyll porphyrins.*—Desoxy-phylloerythrin, which had been synthesized before, was prepared anew (77). By causing it to react for a short time with fuming sulphuric acid containing sulphur, Fischer succeeded in synthesizing phylloerythrin. This substance stands closer to chlorophyll-*a* than any synthetic porphyrin previously prepared (73).



XXIX. Phylloerythrin

By prolonged action of the oxidizing agent, phylloerythrin changes to chloroporphyrin-*e*₅ (γ -formyl-rhodoporphyrin), whereby chloroporphyrin-*e*₄ (phylloporphyrin-6-carboxylic acid) appears as an intermediate product (74).

Further attempts to prepare porphyrins related to derivatives of chlorophyll by oxidation of 6-ethyl-phylloporphyrin (which was synthesized for the first time) and also of the previously unknown γ -methyl-mesoporphyrin, were unsuccessful (75). Equally negative in result were attempts to prepare chloroporphyrin-*e*₄ and porphyrins with oxygen-containing radicals attached to C_γ by direct synthesis from methenes (76).

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THE ALKALOIDS*

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The object of this review is to record progress that has been made during the past biennium in elucidating the molecular structure of the alkaloids.

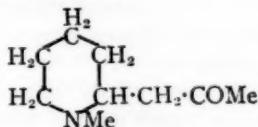
Thus the isolation of new alkaloids from natural sources will not in general be mentioned; nor will the preparation of series of derivatives be described unless they shed fresh light on the constitutional aspect. Synthesis of analogues of the alkaloids is not a part of our subject-matter. Furthermore the topics are selected and no attempt is made to be exhaustive. It is hoped that a series of these reports will effectually cover the field, but certain subjects are held over for consideration until a more suitable time.

Among these, the derivatives of codeine and sinomenine are the most important. Much progress has been made here, but, since there is available the excellent monograph of Small & Lutz (1), the subject may be allowed to mature for another year.

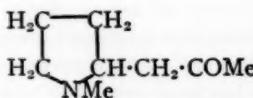
Another topic left for future consideration is the stereochemistry of the alkaloids, concerning which Rabe & Emde have made interesting advances. The chemistry of yohimbine is not ripe for immediate treatment and is likewise relegated.

SIMPLY CONSTITUTED ALKALOIDS

According to Meisenheimer & Mahler (2), methyl-isopelletierine has the formula I, and this has been confirmed by Hess & Littmann (3), who have reinvestigated earlier syntheses. This is of interest in that the parallelism of the piperidine and pyrrolidine groups is maintained. In the present case hygrine (II) is the opposite member in the pyrrolidine series; we compare also ψ -pelletierine with tropinone.



I. Methyl-isopelletierine

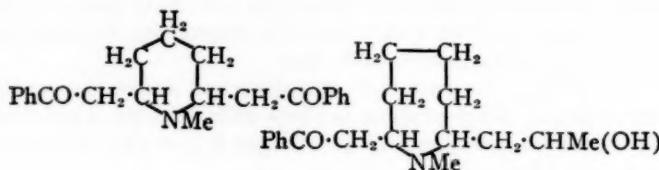


II. Hygrine

* Received February 28, 1933.

It seems obvious that biogenetically the piperidine and pyrrolidine series are related to, possibly derived from, lysine and ornithine respectively. The other fragment is evidently an acetone equivalent.

From this point of view interest attaches to the constitutions of the alkaloids of *Lobelia inflata*, which Wieland and his collaborators have elucidated in the last few years. The five bases, lobelanine, lobeline, lobelanidine, norlobelanine, and norlobelanidine are constructed on the same model (formula III for lobelanine), the variations depending on reduction of one or two carbonyl groups and on the possession of :NH or :NMe as the case may be. However, a new alkaloid of the group, lobinine, appears to be correctly represented by formula IV (4). Unlike the other bases it furnishes no



III. Lobelanine

IV. Lobinine

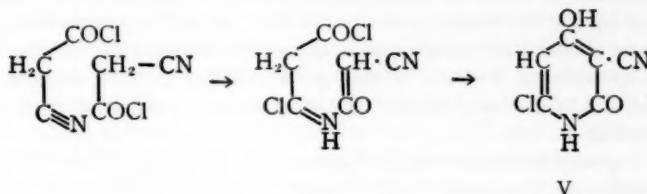
1-methylpiperidine-2: 6-dicarboxylic acid on oxidation; it is a tertiary base, a ketone, and a secondary alcohol. The diketone obtained on oxidation gives a methiodide, which breaks down on treatment with alkalies, giving dimethylamine and an unsaturated diketone, $C_{12}H_{20}O_2$.

This was reduced to $C_{17}H_{24}O_2$ and then, on oxidation, benzoic acid and suberic acid, $CO_2H(CH_2)_6CO_2H$, were obtained. The positive evidence shows that lobinine is IV or an alternative in which the ring is contracted and the side-chain $[CH_2 \cdot CH(OH)Me]$ extended. The negative evidence, the failure to obtain a piperidine-dicarboxylic acid on oxidation, favours IV. In any case the relative positions of the nitrogen and oxygen atoms must remain unaltered. Thus it would appear highly probable that the ring-homologue of piperidine is represented among natural products.

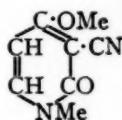
Ricinine.—Although the alkaloid of the seeds of the castor-oil plant has been previously synthesised, a new simple method (5) is noteworthy.

Cyano-acetyl chloride polymerises spontaneously at 5°–8°, producing a mixture in which chloro-*nor*-ricinine (V) predominates.

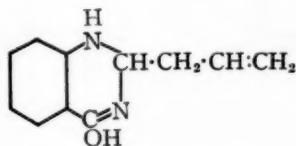
This remarkable transformation is ingeniously attributed to the intermediate formation of malonimide chloride, and cyanoketene.



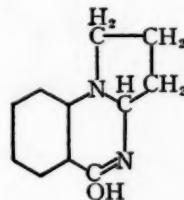
Transformation to ricinine (VI) is merely a matter of reducing out the halogen and methylating the nitrogen and one oxygen atom; the stages need not be detailed.



VI. Ricinine



VII



VIII

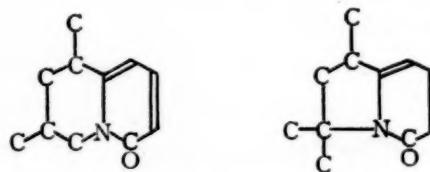
Vasicine.—An attack on the constitution of this simple base, derived from *Adnatoda vasicina* Nees, has been renewed (6). It furnishes 4-quinazolone on oxidation with permanganate and is easily converted by alkaline reagents into *isovasicine*. The formulae VII and VIII have been put forward for these substances, but the reviewer does not find the arguments convincing; somewhat improbable reactions are assumed for the course of the actions of hydrogen peroxide and of acetic anhydride on the parent base. It is, however, very difficult to suggest satisfactory alternatives, and further results will be awaited with interest.

Cytisine.—The alkaloid of the seeds of *Cytisus laburnum* (and a number of other Leguminosae) has fallen a victim to the combined attack of a number of chemists, and its constitution is now known, although the confirmation of synthesis will be welcome.

The *sec-tert*-base has the formula, $C_{11}H_{14}ON_2$, and it is reduced by hydriodic acid and phosphorus (7) with the formation of cytiso-

line and β -cyclosolidine; the former was proved to be 2-hydroxy-6:8-dimethylquinoline (8) and the latter, 6:8-dimethylquinoline (9). Objections to earlier formulae based on these results have been indicated by Ing (10), and especially the fact that no benzene derivative can be obtained on oxidation tells against the assumption common to the formulae of Ewins, Freund, and Späth that cytisine contains a ready-formed, albeit oxidized at one part and reduced at another, quinoline nucleus.

Ing suggested that the skeleton should rather be IX or X, the quinoline nucleus being formed by breakaway of the side-chain from nitrogen, followed by condensation in the β -position of the pyridine ring.

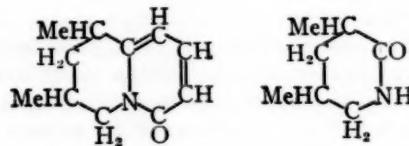


IX

X

Two of the carbon atoms in the system external to the pyridone nucleus are to be joined by an imino-group which is eliminated as ammonia in the formation of cytisoline. A decision between the skeleton formulae IX and X was obtained by a study of the Hofmann degradation of the alkaloid.

Späth & Galinovsky (11) found that the decomposition of methylcytisine methohydroxide proceeded normally only under a very low pressure (0.001 mm.) at 90°. The product was hydrogenated, again submitted to the Hofmann process, and again hydrogenated, yielding tetrahydro-hemicytisylene (XI):



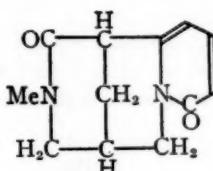
XI. Tetrahydro-hemicytisylene

XII

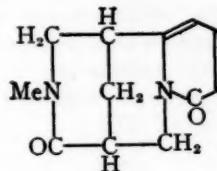
The constitution of this substance was clearly proved, on the one hand by reduction and then oxidation to glutaric acid, and on the other by ozonolysis to the lactam (XII) which could be oxidized to a mixture of the stereoisomeric $\alpha\alpha'$ -dimethylglutaric acids.

These experiments clearly prove that the skeleton IX is correct, and several formulae for cytisine itself come under consideration.

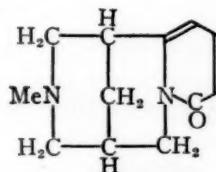
Ing has supplied the necessary discriminatory evidence (12) in the formation of isomeric N-methylcytisamides (XIII and XIV) by the oxidation of methylcytisine by means of barium permanganate. The production of these isomerides affords a neat proof that cytisine (once skeleton IX is accepted) must have the constitution XV.



XIII



XIV



XV. Cytisine

A brief inspection of the possibilities will show that other modes of development of IX into a full structure do not allow of two methylene groups adjacent to the more basic nitrogen atom of methylcytisine. Ing gives convincing reasons for his rejection of the view that the isomeric methylcytisamides are geometrical isomerides.

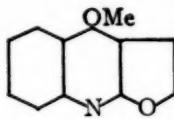
Simple quinoline bases.—Späth & Píkl (13) made the interesting observation that the lesser alkaloids of angostura bark include quinoline, quinaldine, 2-n-amyloquinoline and 2-keto-1-methyl-1:2-dihydroquinoline.

Schöpf & Lehmann (14) have studied the appropriate conditions for the preparation of the 2-alkylquinolines by the Friedländer synthesis.

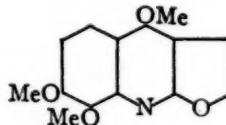
The very interesting and significant point emerges that, at pH 13, *o*-aminobenzaldehyde and hexoylelactic acid give 2-*n*-amylquinoline-3-carboxylic acid, but at pH 7-9, 2-*n*-amylquinoline is obtained in 70 to 75 per cent yield. Other cases of the same kind were observed, and this remarkable influence of pH on the extent of decarboxylation in processes probably analogous to the photosynthetic must be followed up. The reactions were carried out at 25° or at a lower temperature.

Dictamnine and skimmianine.—Though falling somewhat earlier than the period under review, a brief mention of these unusually constituted substances may be permitted.

Dictamnine from *Dictamnus albus* and *Skimmia repens* has been proved by Asahina and his collaborators to be a quinoline-furan (XVI), while skimmianine from *Skimmia japonica* is its dimethoxy-derivative (XVII).

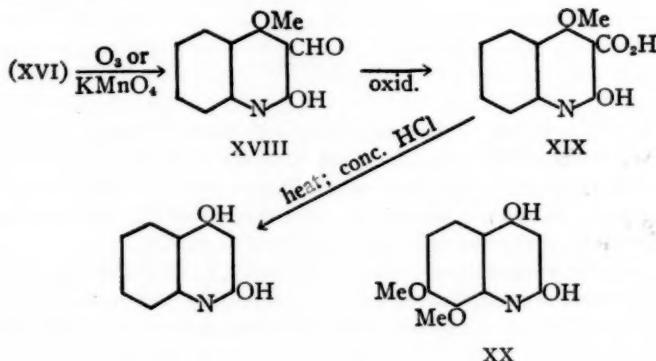


XVI. Dictamnine



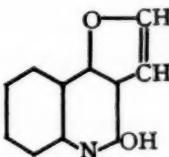
XVII. Skimmianine

The following scheme (15) summarizes the evidence in regard to dictamnine.

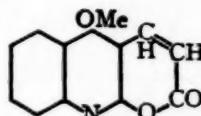


Dictamnic acid (XIX) is not identical with synthetic 2-methoxy-4-hydroxyquinoline-3-carboxylic acid. The degradation of skimmianine (16) was similar, the final product (XX) having been synthetically prepared. Dictamnal (XVIII) can be demethylated by means of hydrobromic acid in acetic-acid solution to *nordictamnal*, which is identical with synthetic 2:4-dihydroxyquinoline-3-aldehyde (17).

Using the well-known coumarin-coumarilic-acid synthesis, this aldehyde furnishes ultimately an *isonordictamnine* (XXI).



XXI



XXII

It is instructive to observe that this route to a synthesis of dictamnine breaks down because the coumarin (XXII) from dictamnal cannot be brominated in the normal manner.

THE LUPININE-SPARTEINE GROUP

The writer is indebted to Mr. G. R. Ramage for assistance in compiling this section of the review.

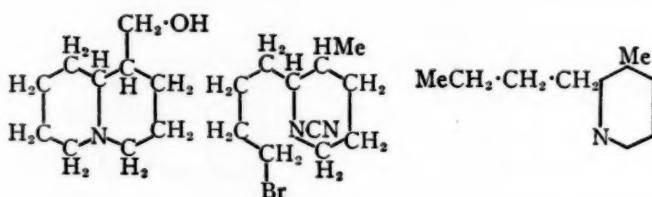
There are five main alkaloids of seeds of species of *Lupinus*, namely, lupinine, $C_{10}H_{19}ON$, and sparteine, $C_{15}H_{26}N_2$, from *L. luteus* and *L. niger*; lupanine, $C_{15}H_{24}ON_2$; hydroxylupanine, $C_{15}H_{24}O_2N_2$; and spathulatine, $C_{33}H_{64}O_5N_4$ (18). Of these, lupinine has been the most intensively studied in recent years and with results that go far to establish its constitution.

Willstätter & Fourneau (19) showed that *l*-lupinine is a saturated tertiary-base containing a primary alcoholic group. They obtained lupinic acid, $C_9H_{16}N \cdot CO_2H$, by means of chromic acid and dehydrated the alkaloid to the unsaturated anhydrolupinine, $C_{10}H_{17}N$. The results of Hofmann degradation proved that the nitrogen was a member of two ring systems.

Karrer and his co-workers (20) repeated the exhaustive methylation but reduced the product of each stage and thus isolated a satu-

rated alcohol, $\text{RCH} \cdot \text{CH}_2\text{OH}$, which by the stages $\text{RCH} \cdot \text{CH}_2\text{Br}$, $\text{RCH} \cdot \text{CH}_2\text{NHMe}$, $\text{R} \cdot \text{CH} \cdot \text{CH}_2\text{NMe}_3 \cdot \text{OH}$ was converted into an unsaturated hydrocarbon $\text{RC} = \text{CH}_2$ ($\text{R} = \text{C}_8\text{H}_{16}$). The latter afforded *n*-propyl *n*-amyl ketone on treatment with ozone.

The identification of this ketone was the subject of some discussion (21), but, this having been cleared up, the correctness of the formula (XXIII) of Karrer became highly probable.

XXIII. *L*-Lupinine

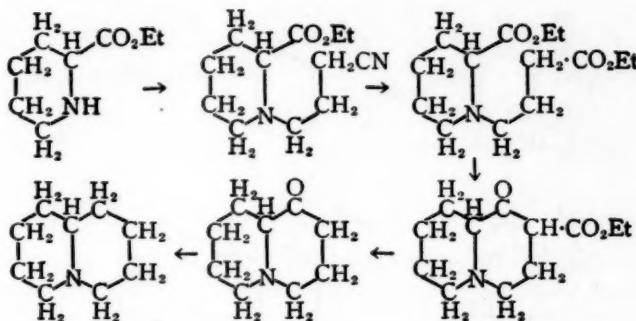
XXIV

XXV

The most striking confirmation of this view was provided by Winterfeld & Holschneider (22, 23), who worked with lupinane, obtained by hydrogenation of anhydrolupinine. Following von Braun they treated this base with cyanogen bromide and thus broke the ring, forming bromolupinane-cyanoamide (XXIV). The bromine was replaced by hydrogen (with some difficulty—hydrogen and palladised calcium carbonate were employed) and the cyano-group eliminated by hydrolysis; the resulting *sec*-base was then oxidized by means of silver acetate to a mixture of pyridine bases $\text{C}_{10}\text{H}_{15}\text{N}$.

There is some dubiety at this stage, and criticisms were forthcoming from more than one quarter. These concerned the homogeneity of the starting products, but it seems certain that the bases $\text{C}_{10}\text{H}_{15}\text{N}$ contained mainly 3-methyl-2-*n*-butylpyridine (XXV) as shown by the formation of 3-methylpyridine-2-carboxylic acid and quinolinic acid on permanganate oxidation.

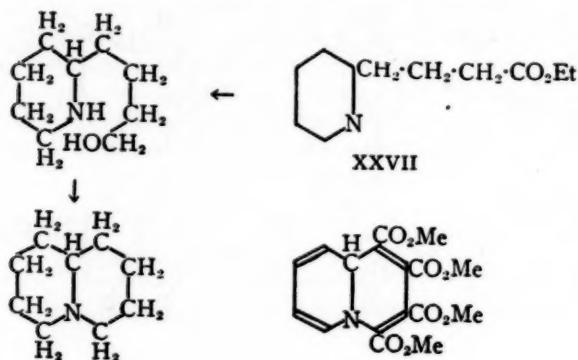
Novel results have been obtained by Clemo & Ramage (24), who have synthesised bases containing the lupinine ring-system, that is, octahydro-pyridocoline (XXVI). Ethyl-piperidine-2-carboxylate was condensed with γ -bromobutyronitrile, the product hydrolysed, esterified, converted into a cyclic ketone by means of sodium, and the product again hydrolysed and reduced by Clemmensen's method.



XXVI. Octahydro-pyridocoline

Now it is remarkable that this synthetic octahydro-pyridocoline did not prove to be identical with *norlupinane*, made, in effect, by taking the elements of formaldehyde from lupinine. Ultimately the conclusion was reached that the synthetic base and *norlupinane* are stereoisomerides of the *cis-trans* decalin type with the interesting feature that a trivalent nitrogen atom is implicated. Before this became clear, however, both *norlupinane* and the syntheses were very carefully scrutinized. The earlier methods for the degradation of lupinine to *norlupinane* having been drastic, Clemo, Ramage & Raper (25) applied the Curtius reaction to lupinic acid. The resulting amino-*nor-lupinine* gave *d-norlupinene* on treatment with nitrous acid and this on catalytic reduction furnished the known *norlupinane* (optically inactive) obtained from lupinic acid by soda-lime distillation. Evidently molecular changes do not account for the anomaly of the non-identity of natural and synthetic products.

Ultimately it was found possible to synthesise *norlupinane* identical with the natural product, and this was independently accomplished by Winterfeld & Holschneider (26) and Clemo, Ramage, and Raper (27). It is perhaps to be noted that both methods depended on a final ring closure at the nitrogen atom; thus the latter authors submitted ethyl γ -2-pyridylbutyrate (XXVII) to vigorous reduction with sodium and alcohol. Replacement of hydroxyl by bromine and liberation of the base from its salts completed this elegant series of reactions.

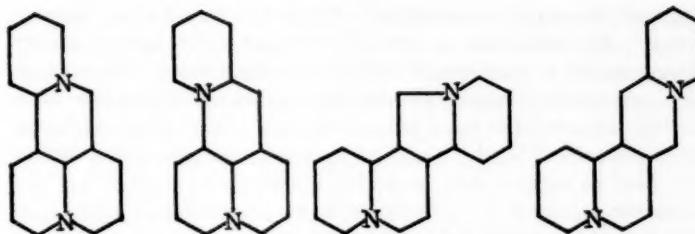


XXVIII. Methyl quinolizine-tetracarboxylate

Diels & Alder (28) have recorded that pyridine and methyl acetylene-dicarboxylate furnish an adduct, which is regarded as methyl quinolizine-tetracarboxylate (XXVIII).¹ Hydrolysis with aqueous potassium hydroxide furnishes quinolizine-dicarboxylic acid, and from this quinolizine itself is obtained by heating with lime. The base forms an octahydro-derivative which should of course be identical with one of the octahydro-pyridocolines. The picrate of the Diels-Alder base has a melting-point of 203° , whereas the picrates of the two pure isomeric bases have melting-points of 194° and 213° respectively.

Attention has also been paid to the chemistry of sparteine and lupanine. A relation between these has been brought to light by the work of Clemo, Raper & Tenniswood (29), who resolved *dl*-lupanine by crystallization of its camphorsulphonate from acetone. The reduction of *d*-lupanine by means of hydriodic acid and red phosphorus then afforded *l*-sparteine. The relation between these bases and lupanine is not so apparent, however probable it may be on general grounds that the sparteine skeleton is that of the C_{10} -series, plus a piperidine nucleus. The four skeletons annexed have been given by Karrer as a concrete expression of this view of the nature of sparteine.

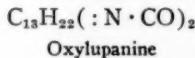
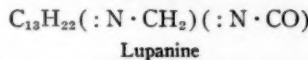
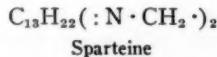
¹ The formula is misprinted in *J. Chem. Soc.*, p. 2962 (1932).



Winterfeld, Kneuer & Holschneider (30) submitted lupanine to very drastic treatment with hydriodic acid and phosphorus and obtained β -lupinane and an oily product giving the pine-shaving reaction. Although it has been suggested that the production of β -lupinane in these experiments was due to contamination with lupanine, the accuracy of the investigation has been reaffirmed. It is to be hoped that general agreement in regard to this point may be reached.

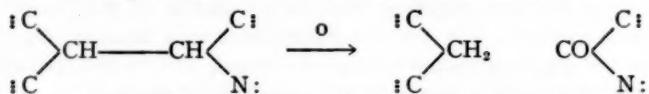
Karreir and his co-workers (31) submitted sparteine to the process of exhaustive Hofmann methylations and catalytic hydrogenation of the unsaturated intermediate products and obtained ultimately a saturated hydrocarbon $C_{15}H_{32}$. Unfortunately the physical characteristics of the various likely isomerides differ so little that it was impossible to identify the hydrocarbon. The three pentadecanes corresponding to the four skeletons (above) were synthesised in the course of this investigation.

Oxidation experiments have not yet given very precise information. Winterfeld (32), using mercuric acetate in dilute acetic acid solution, converted sparteine, $C_{15}H_{26}N_2$, into $C_{15}H_{24}N_2$ and, at a higher temperature, $C_{15}H_{22}N_2$. More promising perhaps are the experiments with lupanine which Clemo & Leitch (33) oxidized by permanganate to oxylupanine, $C_{15}H_{22}O_2N_2$. The composition change is $CH_2 \rightarrow CO$ and as neither lupanine nor oxylupanine gives any reaction for the carbonyl or hydroxyl groups it seems probable that the following relations hold:



It should be noted that sparteine is definitely a diacid base; lupanine forms a dihydrochloride in ethereal solution but this is very readily dissociated to a mono-hydrochloride—it gives only a monomethiodide; oxylupanine furnishes a chloroplatinate (not improbable for a bicyclic diamide) but has a neutral solution. The further oxidation of oxylupanine (34) by means of aqueous permanganate at 40° to 50° adds an oxygen atom producing a compound, $C_{15}H_{22}O_5N_2$ (an isomeride accompanies oxylupanine in its preparation). Hydrolysis with baryta in sealed tubes affords glutaric acid and other compounds, among them a crystalline substance $C_{10}H_{20}O_4N_2$.

This introduction of oxygen without removal of hydrogen is not unique and may denote the break of a bond as in the scheme:



The further development of this attack will doubtless throw much fresh light on the subject, but in the meantime it cannot be conceded, as Clemo and his co-workers claim, that the results are inexplicable on the basis of Karrer's skeletons. There is no positive argument leading to the exclusion of these, and the reviewer's difficulty is that the data admit of too many explanations instead of too few. Finally reference may be made to the fact that sparteine has been found in considerable quantity in technical chelidонine residues (35) extracted from *Chelidonium majus* (Papaveraceae). If this is confirmed it must be recognized that the distribution of sparteine extends beyond the Leguminosae.

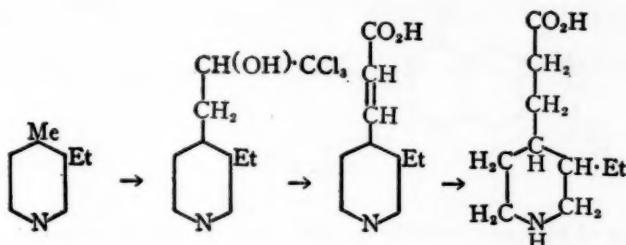
QUININE

As already stated a discussion of the stereochemistry of the alkaloids is postponed.

A useful review of progress toward the synthesis of quinine has been contributed by Kindler (36). The main outlines of Rabe's synthesis of dihydrocinchonine and dihydroquinine were published in 1920-22 (37), but it appears that the whole series of processes has now been carried through with a specimen of ethyl β -3-ethyl-4-piperidyl-propionate, prepared synthetically and resolved by crystallization of its tartrate.

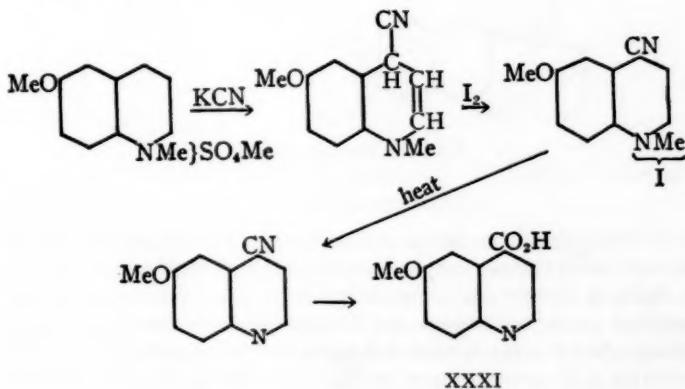
This renders the synthesis of hydroquinine complete, and it is interesting to note how many different groups of workers contributed to this satisfactory outcome.

The pyridine synthesis is that of Thorpe & Rogerson (38), and the particular application to the synthesis of β -collidine (XXIX) was made by Ruzicka & Fornasir (39). Then Königs & Ottmann (40) converted β -collidine into homocincholoipon (XXX), the ethyl ester of which is mentioned above as having been resolved by Rabe and collaborators.

XXIX. β -collidine

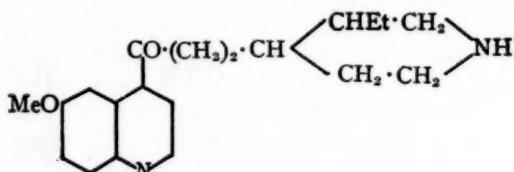
XXX. Homocincholoipon

A choice is available for the synthesis of 6-methoxy-cinchoninic acid (XXXI), one of the most interesting methods being that of Kaufmann & Peyer (41), who treated 6-methoxy quinoline methosulphate with aqueous potassium cyanide and oxidized the product with iodine. The resulting methiodide was distilled in a vacuum and the nitrile hydrolysed.



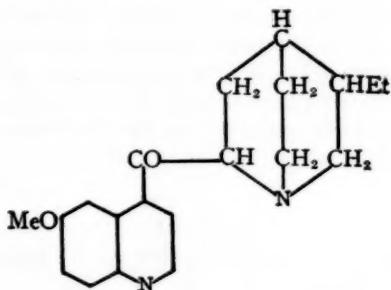
XXXI

Rabe and his co-workers convert the dextrorotatory ethyl ester of homocincholoipon into its N-benzoyl derivative and then couple with ethyl quininate (ester of XXXI) with the help of sodium ethoxide. The product is hydro-quinotoxin (XXXII), and this is converted in the familiar manner (N-bromination or C-bromination and elimi-



XXXII

nation of hydrogen bromide from the product) into hydroquininone (XXXIII). Hydrogenation of crystalline hydroquininone affords only hydroquinidine, but the mixture of tautomerides of hydroquininone affords hydroquinine also.

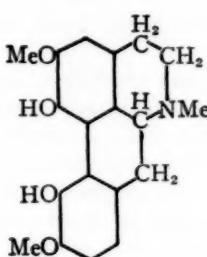


XXXIII. Hydroquininone

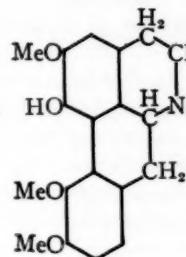
APORPHINE GROUP

The question as to the exact constitution of corytuberine and its isomeric methyl ethers, corydine and *isocorydine*, has been cleared up by Späth & Berger (42), the method being the examination of the oxidation products of mono- and di-ethyl ethers of the bases. Corytuberine diethyl ether furnished 4-methoxy-3-ethoxy-phthalic acid on oxidation with permanganate; the use of a limited amount of diazo-

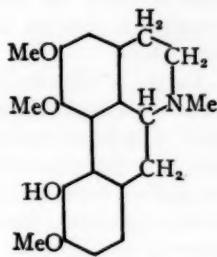
ethane gave a mixture of mono-ethyl ethers and on oxidation these afforded 4-methoxy-3-ethoxy-phthalic acid and also 5-methoxy-4-ethoxybenzene-1:2:3-tricarboxylic acid. Corytuberine dimethyl ether has been synthesised and these facts prove that corytuberine is XXXIV.



XXXIV. Corytuberine

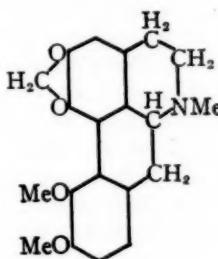


XXXV. Corydine

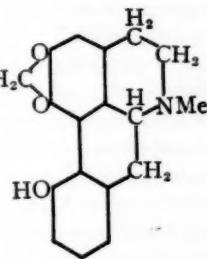


XXXVI. Isocorydine

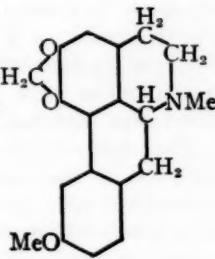
Bulbocapnine-methyl ether is known from synthesis (43) to be correctly represented by the formula XXXVII.



XXXVII



XXXVIII. Pukateine



XXXIX. Laureline

Removal of the methylenedioxy-group by means of sulphuric acid and phloroglucinol furnishes, therefore, a catechol derivative of known constitution; partial methylation is now found to produce corydine which must be XXXV. Isocorydine, again, must be XXXVI, because it is formed together with corydine by the partial methylation of corytuberine.

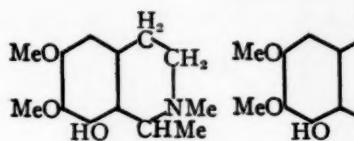
Several groups of workers have been engaged in the direct synthesis of phenolic aporphines (44) and in most cases the benzyl group is selected for the purposes of protection of hydroxyls; this group can be readily removed by hydrolysis under conditions that do not produce demethylation. In all these syntheses the Gulland-Haworth modification of the Bischler-Napieralski reaction is of primary importance; it consists in the use of phosphorus pentachloride for ring-closure.

Barger & Girardet (45) have isolated three new alkaloids of this group from the bark of *Laurelia Novae Zealandiae*, namely, pukateine (a hydroxymethylene-dioxyaporphine), laureline (a methoxy-methylene-dioxyaporphine which is not pukateine methyl ether), and laurepukine, regarded as a dihydroxymethylene-dioxyaporphine. The results of oxidative and other degradative investigations showed that pukateine and laureline were probably to be represented by the formulae XXXVIII and XXXIX respectively. In particular, oxidation of a derivative of pukateine (in which the isoquinoline ring was first broken) by means of potassium permanganate in acetone solution afforded carboxyhydrastic acid, and on heating this substance isohydrastic anhydride was obtained. The underlying cause of the difference between laureline and pukateine-methyl ether was also detected as the result of permanganate oxidations of these substances or of their methines. Pukateine-methyl ether gave rise to 3-methoxyphthalic acid; laureline to 4-methoxyphthalic acid. These constitutional formulae were fully confirmed by syntheses of *l*-pukateine-methyl ether (46) and *l*-laureline (47), following the usual methods.

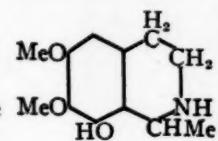
The constitution of laurepukine (48) has not yet been completely elucidated. If the formula attributed to it on the basis of spectrochemical and other evidence is correct, then it bears little relation to its congeners. The methylenedioxy group is placed in the lower nucleus (oriented as XXXVII).

FURTHER TYPES OF ISOQUINOLINE ALKALOIDS

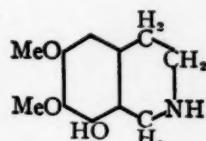
The very simple isoquinoline bases from various Cactaceae are particularly illuminating biogenetically, and hence the exact determination of the structure of the partly methylated members of the group is valuable ground gained. Späth & Passl (49) have continued former work and have proved by the method of ethylation and subsequent oxidation that pellotine is XL and anhalonidine is XLI; it was also rendered probable that anhalamine is XLII.



XL. Pellotine

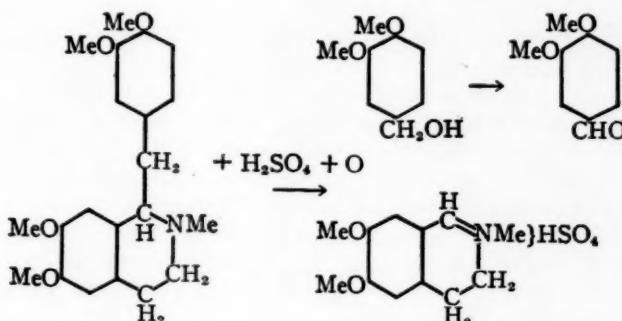


XLI. Anhalonidine



XLII. Anhalamine

The mechanism of the oxidation of laudanosine, and hence of other similarly constituted isoquinoline bases has been indicated by Bhagwat, Moore & Pyman (50), who show that the by-product is tetramethoxy-dihydro-anthracene. This must arise from veratrylcarbinol (51), and hence the process should be represented as follows:

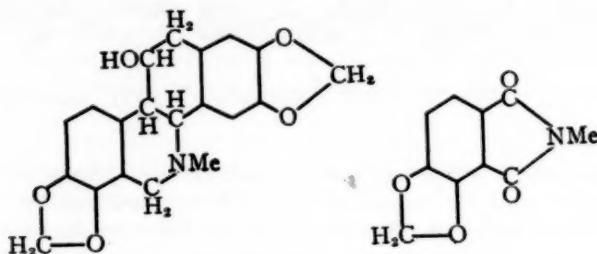


Two stereoisomeric inactive hydrastines have been synthesised (52) by condensation of hydrastinine with nitromeconine, reduction of the product to isomeric aminohydrastines, and removal of the amino-group by way of the hydrazines. Only one of the hydrazinohydrastines gave on oxidation a by-product, dedihydrohydrastine, which is probably a phenanthrene derivative.

The writer may be permitted to add that the synthetic hydrastine has been equated with material made by racemisation of the natural base.

The chelidone-chelerythrine group has received considerable attention and some of the main features are now plain. Bruchhau-

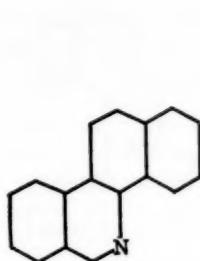
sen & Bersch (53) were the first to assign an acceptable structure to chelidonine (XLIII) :



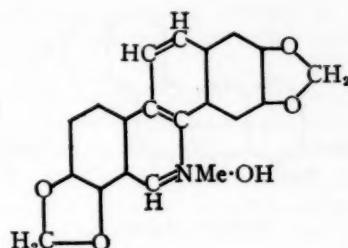
XLIII. Chelidonine

Further evidence in favour of their view has been brought forward by Späth & Kuffner (54).

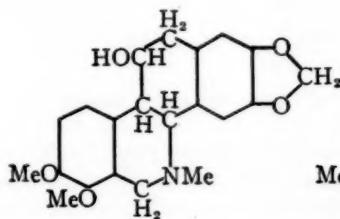
Acetylation of chelidonine was found by Gadamer (55) to involve dehydration and attack of the nitrogen atom; the resulting N-acetylanhydrochelidonine yields benzene-1:2:4-tricarboxylic acid on oxidation with nitric acid. Permanganate oxidation of chelidonine affords hydрастic and *isohydрастic* acids. The validity of the assumed position of the hydroxyl group depends on the effect of cumulative evidence, but it is certainly not in the pyridine ring; the relative position of the methylenedioxy groups and the pyridine ring is indicated by the observed oxidation of chelidonine to 3:4-methylenedioxyphtalmethylimide. Confirmatory evidence is derived from the recognition that a base first obtained by Gadamer from crude sanguinarine can be also obtained from the pure alkaloid and is identical with α -naphthaphenanthridine (XLIV). [It is impossible to overlook the formal constitutional relation to chrysene obtainable from sterol and bile-acid derivatives by drastic processes.] The established facts regarding the relations of chelidonine, sanguinarine, homochelidonine, and chelerythrine have now been supplemented as follows: chelerythrine gives α -naphthaphenanthridine with zinc dust, and on oxidation with permanganate it yields hemipinmethylimide and hydрастic acid. Finally the methylenedioxy groups were removed from dihydrochelerythrine and dihydrosanguinarine and identical bases obtained on methylation.



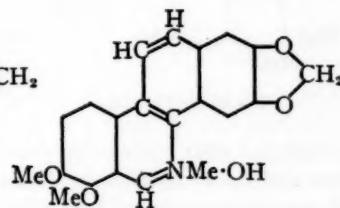
XLIV



XLV. Sanguinarine



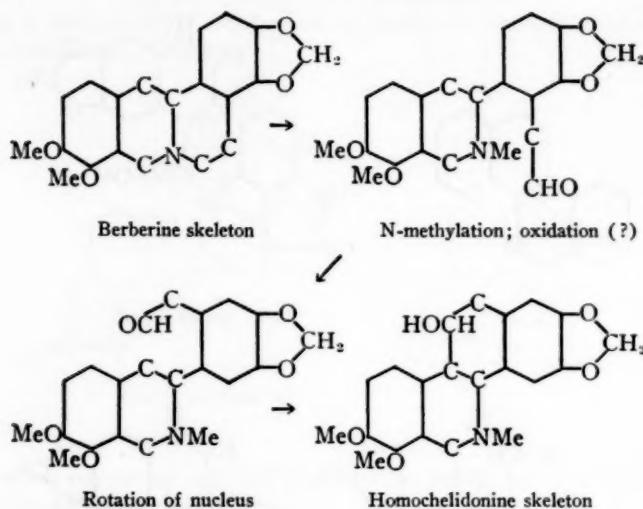
XLVI. Homochelidonine



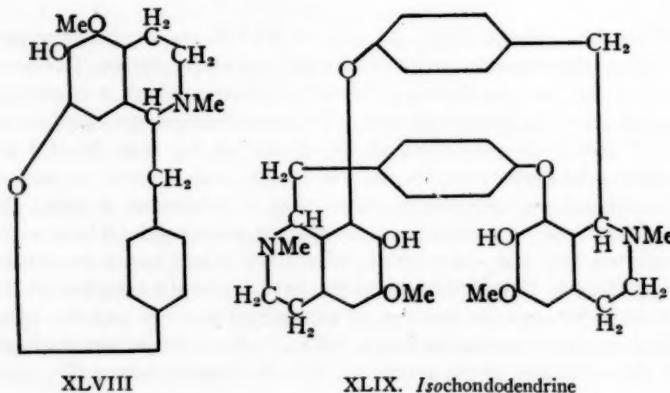
XLVII. Chelerythrine

Hence the formulae XLV, XLVI, and XLVII are attributed respectively to sanguinarine, homochelidonine, and chelerythrine. The latter two, it will be seen, bear a relation to berberine which it is perhaps worth while to express in a scheme of possible biogenetic significance.

It will be recalled that, in the course of his very detailed researches on cryptopine, W. H. Perkin, Jr., was the first to prepare members of the *epiberberine* series; that is, substances in which the methoxyl and methylenedioxy groups are transposed. It was to be expected that the *epiberberine* orientation would occur in natural products, and Goto & Sudzuki (56) have found the canadine of the group in *Sinomenium acutum*. It was termed sinactine and was identified as *l*-tetrahydro*epiberberine* (57); confirmation is now available in the resolution of the synthetic, optically inactive base (58).

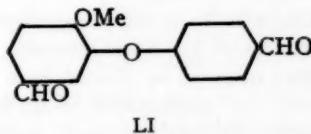
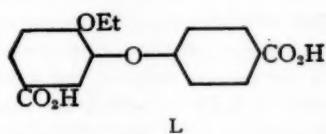


Diphenyl ether types.—The importance of this group continues to grow, and the recognition by Faltis of the occurrence of the ether link was an important step of which his successors have reaped the advantage. The earlier formulae, for example XLVIII for *isochondodendrine* (*isobeebeerine*), contained only one laudanosine nucleus. These are now being replaced by bimolecular formulae, for example XLIX for the base mentioned (59).

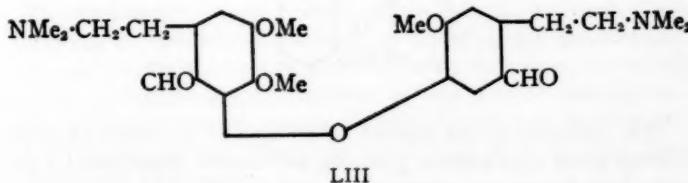
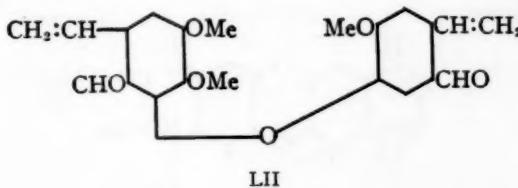


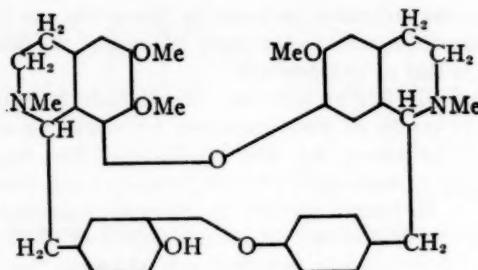
Many of the structures proposed in this group can only be regarded as approximations to the truth, but one of the better understood cases is that of oxyacanthine.

In 1929 Späth & Píkl (60) and Bruchhausen & Schultze (61) published the results of their respective investigations and arrived independently at almost the same conclusions. The base contains three methoxyl groups and a phenolic hydroxyl; this was ethylated and, after a Hofmann degradation process, oxidation afforded 3-phenoxy-4-ethoxybenzene-1:4'-di-carboxylic acid (L). This fixes the position of the phenolic hydroxyl and, of course, that of one of the aryl-ether linkages.



The use of ozone for the oxidation of the methine leads to the production of the dialdehyde (LI), but there is also produced a base, $C_{25}H_{34}O_6N_2$, which forms a dimethiodide (62). Treatment with alkali now results in the production of two molecules of trimethylamine and a neutral, doubly unsaturated, dialdehyde. The latter is LIII, the tertiary base is LIII, and oxyacanthine LIV (p. 440), but the disposition of the upper ether group and the methoxyls is undetermined.

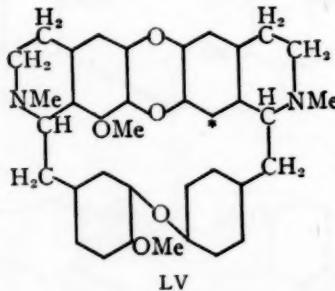




LIV. Oxyacanthine

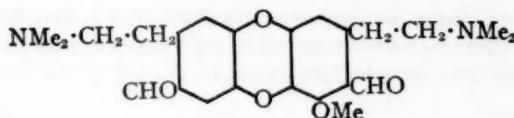
Kondo & Yano (63) have found that tetrandrine, $C_{38}H_{42}O_6N_2$, an alkaloid of *Stephania tetrandra*, gives results entirely comparable to those obtained by Bruchhausen with methyloxyacanthine; the same series of degradation products were isolated. Tetrandrine and methyloxyacanthine would appear to differ only in the relative arrangement of the upper and lower layers of the formula as printed. For example a methyloxyacanthine formula would be converted into a tetrandrine formula by rotating the entire section of the molecule, including the lower phenyl-ether group and the methylene groups. This constitutional peculiarity resides in the unsymmetrical character of both main sections of the molecule.

According to Kondo & Tomita (64), trilobine and isotrilobine (homotrilobine) are similar isomerides (LV), the formula representing one of the bases and the other bearing the methoxyl at the asterisked position.



LV

The oxidation of the methine of either base by means of ozone in dilute acetic acid solution gives the well-known dialdehyde LI' and as the basic fragment a substance regarded as LVI:



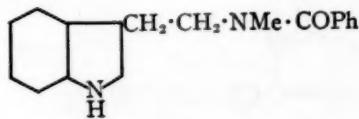
LVI

The side-chains were reduced to four carboxyls, but full degradation then became difficult; protocatechuic acid and possibly gallic acid were obtained by potash fusion. However, the nature of the substance and therefore of these complex alkaloids is established in its main features; certainly a remarkable achievement of combined attack on the part of Faltis, Bruchhausen, Späth, and Kondo.

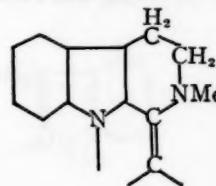
THE INDOLE GROUP OF THE ALKALOIDS

The close relation of calycanthine to what may well be called the tryptophane group of natural products has long been suspected, but Manske (65) has established this clearly by benzoylating the alkaloid and then degrading it to benzoyl-N-methyltryptamine (LVII) by oxidation with permanganate, followed by treatment with alcoholic potash. The *p*-nitrobenzoylation of calycanthine afforded some of the *p*-nitrobenzoyl-N-methyltryptamine directly.

Formerly calycanthine was thought to have the formula C₁₁H₁₄N₂, but a double molecular formula is now considered more probable and Manske suggests LVIII as a part structure.



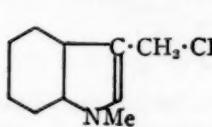
LVII



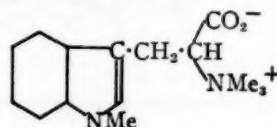
LVIII

The reviewer is of the opinion that a possible alternative is the coupling of two tryptamine units by means of the unsaturation of the pyrrole nuclei. Carbon attached to the α -position of the indole group in carboline derivatives is not so readily removed as in Manske's experiments; on the other hand, indole derivatives with a free α -position result from the breakdown of even quite heavily substituted hydroindoles.

Wieland, Herse & Mittasch (66) have shown that bufotenine and bufotenidine from the secretions of toads are respectively trimethyl- and tetramethyl-tryptophanes (LIX, LX).



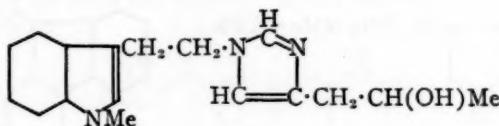
LIX



LX

These interesting substances are not strictly alkaloids, but LX is the N-methyl derivative of the betaine hypaphorine from *Erythrina hypaphora* (67).

On the strength of the observations of characteristic colour reactions, many alkaloids appear destined for eventual recognition as indole derivatives. Unusual interest attaches, however, to the cases of ergotoxine and ergotinine which have been found to give a new base, ergine, $C_{17}H_{21}ON_3$, when submitted to the action of alcoholic potash. Ergine represents about half of the original molecule; it is optically active and mono-acid. It contains a methyl-imino-group and gives colour reactions for both indole and glyoxaline derivatives (68). It is always useful to write down a plausible structure with which an alkaloid is isomeric, and bearing the foregoing in mind it is interesting that ergine is isomeric with LXI.

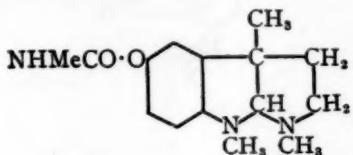


LXI

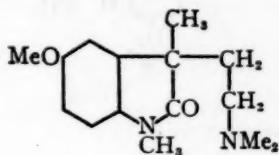
It is hardly necessary to draw attention to *Ergot and Ergotism* by Barger. This exhaustive monograph was universally acclaimed, on its appearance, as a masterpiece of lucidity and scholarship and it is indispensable.

The quaternary carbon grouping attributed to physostigmine (eserine) is doubtless the result of a process of methylation in nature which has its laboratory counterparts; the occurrence of the group has been synthetically confirmed (69). Eserine (LXII) is hydro-

lysed to eserole, then methylated, and the methine oxidized to dehydro-esermethole-methine (LXIII); these processes follow exactly the preparation of the corresponding eserethole derivatives (70).

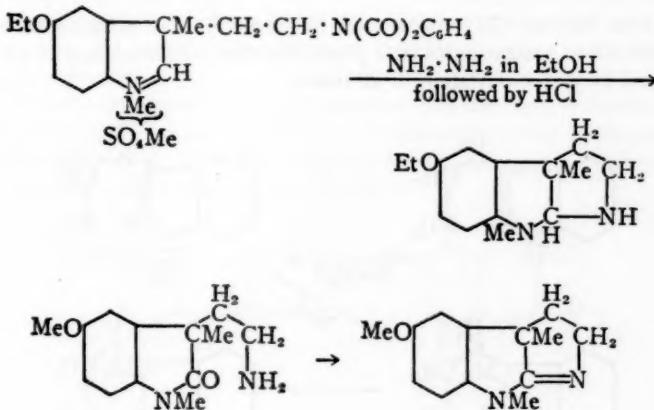


LXII. Eserine



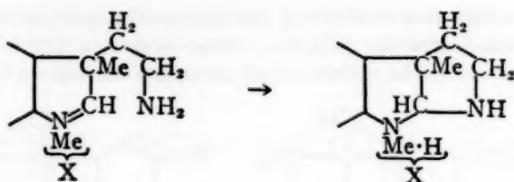
LXIII

Different methods have been utilized for the syntheses of *r*-eserethole and *r*-esermethole (71) and the essential ring-closure is illustrated below.

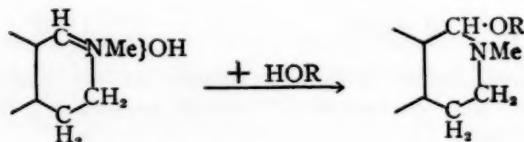


In the first case the base is subsequently methylated to eserethole, and in the second it is methylated and reduced in order to reach esermethole. The completion of the synthesis of eserine requires, therefore, only the optical resolution of the synthetic theseserole or metheserole, combined with a careful repetition of the process of demethylation of metheserole and the conversion of eserole into eserine.

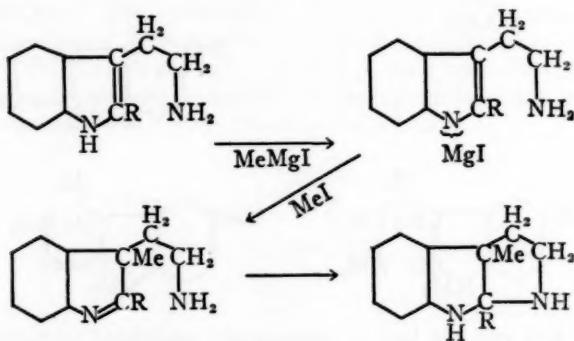
In the first of the above-described processes for closing the eserine ring the underlying reaction is clearly:



analogous to

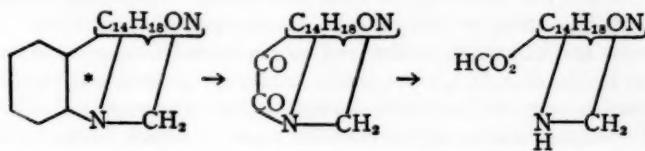


A new method (72) relies upon the same type of addition to an unsaturated system in its later phase, but the indolenine base is produced by direct alkylation of an indole.



The most notable recruits to the indole group of the alkaloids are strychnine and brucine and analogous bases. Although some chemists have been consistent in recognizing that they were probably based on indole, definite proof of this has only recently been obtained. In the first place the development of Leuchs' researches on the breakdown of the aromatic nucleus of brucine (and strychnine) gave results which could only be explained on the assumption that this

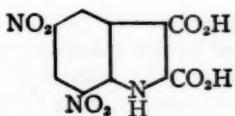
nucleus is fused to a 5-ring (73).² For example strychnidine was oxidized by chromic acid to 2:3-diketonucidine (LXIV), and this becomes carboxy-*α*-ponucidine on further oxidation by means of hydrogen peroxide.



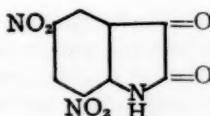
LXIV

If the asterisked ring had been six-membered, there is little doubt that LXIV would yield a pyrrolidone by interaction between the carboxyl and imino-groups. But this is not the case, and neither this amino-acid nor carboxy-*α*-ponucine shows any tendency to lactam formation; we must assume therefore that strychnine is a dihydroindole derivative.

Shortly afterward it was demonstrated that dinitro-strychnolcarboxylic acid, an oxidation product of strychnine obtained by the use of hot dilute nitric acid (74), is a dicarboxylic acid (LXV) and can be degraded (75) to dinitroisatin (LXVI).



LXV



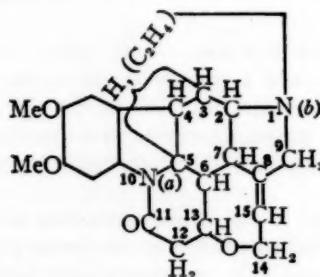
LXVI

Furthermore it may be recalled that the colour reactions of numerous acylated tetrahydroquinoline and dihydroindole derivatives were examined (76) and found to differ; the comparison with corresponding strychnine reactions was heavily in favour of the dihydroindole structure. In 1930 the present reviewer (77) discussed the position of the chemistry of strychnine and brucine, but on the basis that the alkaloids are quinoline derivatives. The greater part of the struc-

² The reference is to the deduction, the experimental work having been done earlier.

tural hypotheses advanced are still applicable, however, and especially the explanation of the course of the permanganate oxidation of brucine and strychnine and the subsequent processes is equally valid on the indole basis.

We owe our knowledge of these transformations, of such fundamental importance to the subject, to the masterly researches of Leuchs, and the writer is flattered by the recent acceptance of his views by the chemist whose opinion carries the greatest weight. On the indole basis the strychnine formula is best expressed as LXVII (78) because there is still a doubtful point in regard to the C_2H_4 chain (brucine is dimethoxystrychnine as indicated).



LXVII. Strychnine

This formula can be justified in detail as follows:

1. The benzene nucleus is required by the researches of Tafel, Leuchs, Wieland, Perkin, and Robinson. The position of the methoxyls in brucine was indicated by a comparison of colour reactions of synthetic quinoline derivatives and brucine, and it was proved by the results of the systematic breakdown of the aromatic nucleus of brucine by Leuchs and Wieland and their respective co-workers.

A confirmation is provided by the oxidation of brucine to N-oxalyl-4:5-dimethoxyanthranilic acid (79).

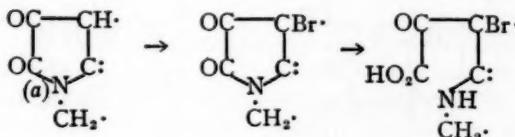
2. The evidence for the indole nucleus is mentioned above.

3. The evidence for the atom-chain $N(a)$ to $N(b)$ including positions 10, 11, 12, 13, the ether oxygen, 14, 15, 6, 7, 8, 9, and 1, is the theme of the reviewer's "Bakerian Lecture" (77) and need not be repeated here. Additional evidence for the methylene group at 12 is, however, available in the preparation of *isonitroso-* derivat-

tives of strychnine and dihydrostrychnine, brucine and dihydrobrucine (80). It is noteworthy that, whereas strychnine and brucine behave normally, the dihydroalkaloids, after nitrosation, suffer fission of the lactam ring (at 10-11) with the formation of, for example, oximinodihydrostrychnic acid.

4. There is no direct evidence for the link 5-6, but the group at 6 is certainly $\equiv\text{CH}$ and not $=\text{CH}_2-$ and hence the link in question is very probable.

5. It appears certain that position 4 bears a hydrogen atom. This conclusion is based on the ready bromination of 2:3-diketonucidine in cold aqueous solution (81). By contrast with the behaviour of related substances the facile attack must be attributed to the influence of a carbonyl group on a neighbouring methine or methylene group. The only carbonyl in question can be that in the β -position to N(*a*) and hence the carbon at 4 must bear a hydrogen atom.



Diketonucidine (part formula)

6. According to the theory of the permanganate oxidation series, brucinonic acid should contain $\text{CO} \cdot \text{CO} \cdot \text{N}$ at positions 8, 9, 1. This has been confirmed by a study of the products of oxidation of the acid with peroxide (81, 82). The results are not quite simple, but it seems to be clear that the expected reaction occurred, and after loss of the carbon at 9 as carbon dioxide the resulting amino-acid group exhibits no tendency to lactam formation.

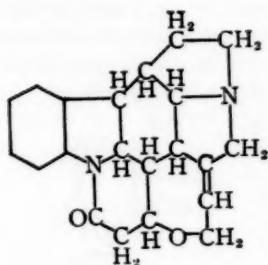
Leuchs claims that this proves that N(*b*) forms part of a 5-ring; in any case it would be difficult to accommodate a 6-ring at this point, especially if the N(*b*) is to be so placed that the molecule contains a tryptophane skeleton.

7. The evidence for the 6-ring (2, 3, 4, 5, 6, 7) is the formation of carbazole from strychnine in numerous reactions, not all at a very high temperature.

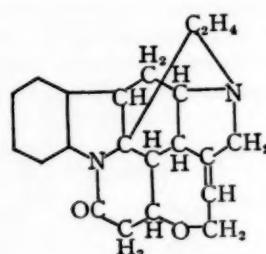
8. There remains the group C_2H_4 which may take the form : CHCH_3 or $\cdot\text{CH}_2 \cdot \text{CH}_2 \cdot$; it must be attached to N(*b*) and to the

carbon at either 3 or 5. If it is attached to position 5 then the ethylidene arrangement is probable and the strychnine skeleton will embody that of harmine (83). If it is attached to position 3, then the ethylene arrangement is certainly correct. There is no positive evidence favouring one view or the other, and while Leuchs prefers LXVIII the writer prefers LXIX because the alkaloids do not appear to have the properties of dihydroindoles.

A considerable amount of detailed work dealing with the derivatives of strychnine and brucine has been published, but in most cases the result has been to formulate new problems rather than to solve the old ones.

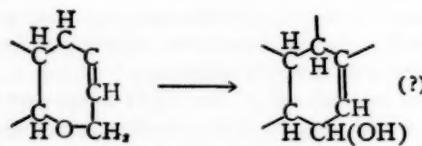


LXVIII

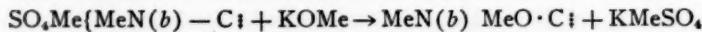


LXIX

There is still no satisfactory explanation of the strychnine-*isostrychnine* change, which involves the appearance of alcoholic hydroxyl and yet leaves the state of saturation unaltered: perhaps the 7-oxide-ring crumples.



The nature of the changes leading to the *neo*-series of alkaloids has been made plain. The metho-salts of strychnine, strychnidine, brucine, brucidine, or their dihydro-derivatives all suffer fission at N(b) when treated with methyl alcoholic potash.

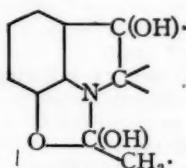


The methoxyl probably attaches itself to C9 and at the same time the double bond (if present) shifts to 8:7. Boiling dilute acids convert the unsaturated substances into (say) methyl*neo*-strychninium salts from which *neo*-strychnine can be prepared by thermal decomposition (84). The saturated methyl-methoxy derivatives can also be made to lose the elements of methyl ether, but with much greater difficulty. These and other degradations round N(b) will be further discussed in a later review, because the writer is cognisant of unpublished work which should be noticed at the same time.

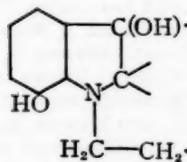
Curious properties characterize some substances which Wieland & Hölscher (85) have obtained by treating strychnos bases with Grignard agents. Apparently the :N(a)·CO·CH₂· group becomes :N(a)·CR=CH· and at the same time :N(b) is alkylated; the products are thus quaternary ammonium salts. They are readily discoloured even after perhydrogenation, and Wieland & Hölscher find in this behaviour an objection to the constitutional formulae discussed above.

The colour reactions of strychnidine and tetrahydrostrychnidine, as well as of analogous bases, on oxidation in acid solution, are attributed by Wieland, Calvet & Moyer to the formation of diphenoquinone-di-imonium compounds. Thus strychnidine is converted through a red dye into distrychnidyl by coupling the aromatic nuclei in the *p*-position to N(a) (86). On this basis Leuchs & Beyer (87) have been able to explain the puzzling results of the oxidation of tetrahydrostrychnine with chromic acid. Coupling occurs to distrychnyl and then one of the residues is oxidised to carboxyl and the product is strychnine-*p*-carboxylic acid.

The investigation of vomicine has been actively continued by Wieland and his co-workers (88), and the results are now considered to be best explained by a part formula LXX for vomicine and LXXI for vomicidine.



LXX. Vomicine



LXXI. Vomicidine

Three new strychnos bases have been isolated from technical mother liquors by Warnat (89); they are the α - and β -colubrines and pseudostrychnine. The colubrines are the isomeric monomethoxystrychnines which can be called de-methoxybrucines; their respective constitutions were demonstrated by oxidation to N-oxalyl-4-(or 5-)methoxyanthranilic acids.

Pseudostrychnine is hydroxystrychnine probably containing the group $>\text{N}(b)\cdot\text{C}(\text{OH}):$. Warnat showed that it condenses with alcohols to form ethers, and it has since been found to furnish strychnine on reduction (83). The methyl ether undergoes the Hofmann degradation to a methine, hydrolysed by dilute acids to methyl alcohol, and a ketone N-methyl-chano-pseudostrychnine which forms a dibenzylidene derivative. This may indicate the existence of a methylene group at position 3 (LXVII) but there is the possibility that the effect of the carbonyl group is transmitted through a double bond to a different methylene.

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CHEMICAL ASPECTS OF PHOTOSYNTHESIS*

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THE PHOTOSYNTHETIC APPARATUS

Aside from the splendid researches on chlorophyll, notably by Hans Fischer and more recently by Conant and others, as well as a beginning of work on the yellow pigments,¹ relatively little has been done toward gaining more intimate knowledge of structure, composition, and properties of the photosynthetic apparatus. There is some indication that, in photosynthesis, surface reactions on the chloroplasts are involved in which iron plays a part. Noak (40) and his students (19, 49) have endeavored to extend the early investigations of Moore on the rôle of iron in photosynthesis. Noak's interpretation of the deleterious effect of removing CO₂ from an illuminated chlorophyllous organ is that under these conditions the photochemical energy is diverted from its normal acceptor, the carbonic acid, to the protoplasm and to the chlorophyll itself, resulting in oxidative destruction of these cell constituents. According to this view the action of substances which inhibit photosynthesis, such as narcotics, is due to a separation of carbonic acid from the chlorophyll on the surface of the chloroplasts, or, in the case of toxic gases, such as sulphur dioxide and cyanide, to a removal or inactivation of the catalytically active iron of the chloroplast. Through the action of toxic gases a portion of the iron becomes water-soluble; in quantity this approaches the amount freed on treating the leaf with boiling water. Noak concludes that the stroma contains simple compounds of iron, loosely bound, and that the action of toxic substances results in the elution of this iron from the stroma. Gassner and Goeze (17) report that the photosynthetic rate of wheat is increased by a lack of potassium and decreased through additions of this element to the mineral nutrient solutions.

Solarization in leaves has been investigated by Holman (25), who

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¹ Recent work in plant pigments has been reviewed by P. Karrer and A. Helfenstein in this publication [1, 551-80 (1932)]. See also Bogert, M. T. (5) and reviews of Willstatter's work [*Naturwissenschaften*, 23, 608 (1932)].

concludes that this is not due to an accumulation of the products of photosynthesis, to water deficit, nor to high temperature, but is probably due to a direct effect of light on the chloroplasts. Solarization could not be obtained in leaves which were starch-filled at the beginning of a period of illumination; increased CO_2 supply retards solarization. Similar photic inhibitions have been described by Montfort and Neydel (37) and named by them "*Sonnenstich-Wirkungen*." Van den Honert (59) suggests that solarization may be due to an inhibition of the dark-reaction in photosynthesis.

The influence of light on the formation of the leaf pigments, chlorophyll, carotene, and xanthophyll, has been studied by Sjöberg (55). In general, strong illumination resulted in active pigment formation, while in weak light this was less. When the natural illumination during winter and spring (in Sweden) was supplemented with electric light, chlorophyll-content was increased. Later in the year this extra illumination had no effect. The results are somewhat at variance with those previously obtained by Guthrie (20), though the conditions were not entirely comparable in the two investigations. The results obtained with periodic illumination indicate that longer periods of low-intensity illumination produce more rapid pigment formation than short periods of high intensity. The ratio of carotene to xanthophyll, c/x , was in general constant throughout the entire period of growth. In *Rubus idaeus* chlorophyll disappeared slowly in autumn, while the carotenoids merely diminished; the c/x increased as the leaves became yellow. When these plants were artificially illuminated the pigment content increased, attained summer values, and the c/x became normal. The carotenoids of leaves, especially xanthophyll, are formed in the dark. On illumination both are increased, although it is difficult to determine to what extent this is a direct influence of light or whether other synthetic processes dependent on chlorophyll, which is simultaneously formed, are involved.

The precursor of chlorophyll, protochlorophyll, from which the green pigment is formed through photo-oxidation, has been investigated by Noak (41) and his students. Although the chemistry of protochlorophyll has not yet been worked out, it has been possible, through reduction of chlorophyll, to obtain magnesium-free compounds which are apparently closely related to the magnesium-free compounds of protochlorophyll. The fundamental difference between protochlorophyll and chlorophyll-*a* seems to lie in the absence of one oxygen atom from the former. Scharfnagel (49) has shown that

protochlorophyll undergoes photo-oxidation to chlorophyll in the living cell. There is some indication that in the cell the same reaction can be brought about in the dark with hydrogen peroxide and with quinone.

The whole subject of chlorophyll formation, especially as to the terms used for the precursors of chlorophyll, is in need of more careful statement. Lubimenko and Hubbenet (35) consider that the greening process takes place in the following stages: (*a*) synthesis of leucophyll, (*b*) transformation of leucophyll into chlorophyllogen, (*c*) transformation of chlorophyllogen into chlorophyll. "The first two reactions occur in the dark, but their course is stopped as soon as the quantity of chlorophyllogen attains some limit quantity. The last reaction is purely photochemical." By use of a spectrophotometric method they found that greening of etiolated wheat seedlings begins at 2–4°, attains its maximum rate at 26–30° and ceases near 48°. Other relations of the effect of temperature on chlorophyll formation were also investigated.

Exact determinations of the optical properties of leaves, especially of the coefficient of absorption, are of importance for the estimation of the energy balance of photosynthetically active leaves. Seybold (54) has determined the transmission of various spectral regions with thermo-electric and photo-electric methods of various green and variegated leaves. The approximate distribution of energy has been calculated as follows:

	White Leaf	Green Leaf
Incident radiant energy.....	100	100
Absorption of white leaf substance.....	40	20
Reflection	30	10
Pigment absorption.....	..	60
Transmission	30	10

The absorption varies, of course, in the different species, but in general the upper epidermis receives ten times the energy of the lower one. Schmucker (50, p. 850) has also indicated the effect on absorption of anthocyanin pigmentation in the lower epidermis.

The diffusion of the CO₂ of the air through perforated septa has been shown by Deneke (10) to increase with movement of the air. Absorption reaches a maximum at about 100 meters per minute. Higher rates of air movement have little effect on the absorption and at this rate the absorption through septa is 85–90 per cent that of an open surface. The effect of air movement on CO₂ absorption by living plants was the same as on perforated septa.

MECHANISM OF THE PHOTOSYNTHETIC REACTION

The mechanism of the photosynthetic reaction and the rôle which the pigments play are still obscure. There appear to be at least two reactions involved, one a dark-reaction—the Blackman reaction—and the other a photochemical reaction. Gordon (18) has suggested the following equations as representing the rôle of chlorophyll.

1. $6C_{55}H_{70}O_6N_4Mg + 6H_2O \rightarrow 6C_{55}H_{72}O_6N_4Mg + 6O_2$
Chlorophyll-*b* + water → chlorophyll-*a* + oxygen
2. $6C_{55}H_{72}O_6N_4Mg + 6CO_2 \rightarrow 6C_{55}H_{70}O_6N_4Mg + C_6H_{12}O_6$
Chlorophyll-*a* + carbon dioxide → chlorophyll-*b* + glucose

A similar scheme has been suggested by Conant, Dietz, and Kamerling (9) on the assumption that chlorophyll is dehydrogenated by carbon dioxide:

1. Dark-reaction
 $12 \text{ Chlorophyll} + 6CO_2 + \text{enzyme} \rightarrow 12 \text{ chlorophyll} (-2H) + C_6H_{12}O_6 + 6H_2O$
2. Light-reaction
 $12 \text{ Chlorophyll} (-2H) + \text{light} + 12H_2O \rightarrow 12 \text{ chlorophyll} + 6O_2$

Emerson and Arnold (16) have studied some of the relationships of the light- and dark-reactions by the use of intermittent illumination on *Chlorella*, much after the manner of Warburg's earlier experiments. From their experiments it cannot be determined whether the light-reaction precedes or follows the dark-reaction, but they conclude that the light-reaction can take place in about 0.0001 second and that the dark-reaction requires less than 0.04 second for completion at 25° and about 0.4 second at 1.1°. The concentration of CO₂ affects the rate of the light-reaction, but seems to have little or no influence on the dark-reaction. It would be of value to have the results of similar experiments with other plants and by the use of other methods for measuring photosynthesis, for in the present state of the subject it is important to have comparative studies in order that individual peculiarities of the organisms used may be revealed. T. H. van den Honert (59) has investigated the influence of light-intensity, of CO₂ concentration, and of temperature on the filamentous alga, *Hormidium*. The curves obtained are in fair agreement with "ideal Blackman curves." The greatest deviations (25 and 30 per cent at the transition-point) were found in the experiments in which the intensity of illumination was varied. Concerning this, van den Honert states:

that even in a single chloroplast much light is absorbed and the different parts of a plastid are submitted to different light intensities. Especially the red light, which is the most valuable for assimilation, is absorbed to a considerable extent. If this assumption holds, light would not become the limiting factor at the same instant throughout the plastid.

The same objection, which is valid for leaves to such a great degree seems not to be entirely eliminated even in a single cell layer. However, this curve agrees much better with Blackman's scheme than the curve obtained by Warburg (1919, fig. 11) with *Chlorella*. I assume, therefore, that in Warburg's experiments a certain number of the algae were still shaded by others.

Van den Honert found at temperatures between 12° and 20°, with high light-intensities, a $Q_{10} = 1.87$, while when light was the limiting factor a $Q_{10} = \pm 1$ was found. When the concentration of CO_2 is the limiting factor he considers that the rate of photosynthesis "is always determined by the diffusion process" and he develops the view that the entire photosynthetic process is a chain reaction "consisting of at least three consecutive processes, viz., a diffusion process, a photochemical process, and a dark chemical process."

With the same organism (*Hormidium flaccidum*) and with some improvements in van den Honert's apparatus, van der Paauw (60) did not obtain typical Blackman light-intensity photosynthetic-rate curves. When the reaction was retarded through the addition of narcotics a logarithmic type of curve was obtained, and it would appear that a variety of factors may exert an influence to modify the shape of the rate curves with regular control of the external factors (temperature, CO_2 , and light). Contrary to the results of Warburg with *Chlorella*, van der Paauw found that the photosynthetic rate of *Hormidium* is stimulated by low concentrations of potassium cyanide and that it was impossible to retard the photosynthetic rate below the compensation-point of respiration and photosynthesis. A parallelism between the rates of photosynthesis and respiration appears in these investigations and van der Paauw revives the idea of a connection of the two in the sense that "there is no direct causality between assimilation and respiration, but a relation between assimilation and some protoplasmic functions, which are, however, (like all vital functions) closely connected with respiration." Van der Paauw claims a connection between photosynthesis and other protoplasmic processes on the grounds of: (a) the equality of the temperature coefficients; (b) the parallel stimulation or retardation through certain chemicals; (c) the parallel and simultaneous recovery from the effect

of these; and (*d*) evidence of a chemical reaction preceding photosynthesis of which the Q_{10} is almost equal to that of respiration. To the reviewer it would appear that such a relationship may depend upon some common agent or force which enables water to be split, $\text{HOH} \rightarrow \text{H} - \text{OH}$.

The photosynthetic activity in different portions of the spectrum has been reinvestigated by Schmucker (50) by the use of light filters and a modification of the bubble-counting method with *Cabomba caroliniana* and *Cryptocoryne ciliata*. The measurements of Warburg showed that the photochemical action is about the same in all portions of the spectrum if the absorption of radiant energy by the chlorophyll is considered. The specific photochemical action decreases from red to blue; in red and yellow light four quanta are required for the reduction of one molecule of CO_2 ; in blue light five quanta are required for the same work. Schmucker obtained essentially the same results as Warburg by entirely different methods and with other plants. Willstätter and Stoll concluded that the light absorbed by the carotinoid pigments was without influence on photosynthesis. Schmucker is of the opinion that the lower photochemical coefficient in blue light (about 15 per cent) is due to the absorption of this light by the carotinoid pigments. This light could be active in photosynthesis, but being absorbed by the carotinoids, is probably lost for this direct purpose. Schmucker showed that contrary to previous findings, the removal of the spectral region absorbed by the carotinoids results in decreased photosynthesis.

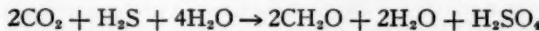
From observations of changes in the brightness of the fluorescence of chlorophyll in leaves, Kautsky and Hirsch (27) have endeavored to interpret the energy relations of the first steps in the chain of reactions comprising photosynthesis. In this preliminary note they state that their results confirm the conclusion that the brightness of the fluorescence of chlorophyll is inversely proportional to the amount of absorbed energy which is converted into chemical work. The difference in fluorescence of chlorophyll under conditions favorable and unfavorable to active photosynthesis are thus explained. The experimental data are promised for a later publication. Padoa and Vita (42) have observed shifts in the spectral absorption bands of solutions of chlorophyll which had been treated with oxygen, carbon monoxide, and carbon dioxide, as compared with pure chlorophyll solutions. From this they conclude that chlorophyll forms labile compounds with these gases.

Photosynthesis in the purple and green sulphur bacteria has been the subject of a careful investigation by van Niel (61, 62), whose conclusions regarding the mechanism of the photosynthetic reactions in the rather unique system obtaining in these organisms is made the basis for the interpretation of the general photosynthetic reaction. These organisms require hydrogen sulphide and light for their development. The purple bacteria can also utilize elementary sulphur, sulphites and thiosulphates, and it is pointed out that oxygen is not liberated by either the purple or the green forms. The problem has been in what manner the oxidation of the H₂S is involved in the reduction of the CO₂; is there a chemosynthesis of organic matter from CO₂ through the energy derived from the oxidation of H₂S, or does the H₂S play some other rôle? It is pointed out that in the experiments, which were carried out anaerobically, "in order to obtain sufficient oxygen for the oxidation of 12 molecules of H₂S the organism would have to reduce 24 molecules of CO₂ by a photochemical process. The quantity of oxygen thus obtained would be sufficient for a chemosynthetic process during which only 1 mol. of CO₂ could be reduced chemosynthetically, producing 0.3 mg. of organic matter." Actually the amount of organic carbon synthesized is more than 15 times greater than can be accounted for on the basis of a chemosynthetic process. In explanation of the mechanism of the reaction van Niel adopts the oxidation-reduction principle of Wieland as applied by Kluyver and Donker to metabolic reactions. Thus in these organisms H₂S is the hydrogen donor, the CO₂ the hydrogen acceptor, and the reaction may be expressed:



The rôle of the green pigment, which in some form exists in purple and green sulphur bacteria as well as in green plants, is that of a photochemical activator of the CO₂ molecule.

The green sulphur bacteria dehydrogenate the H₂S only to sulphur. With the purple sulphur bacteria the H₂S is completely converted to H₂SO₄, no CO₂ reduction occurs when there is no H₂S or S, and the quantitative relations correspond to:



It is proposed that this is "one of a number of possible photosynthetic reactions of the general type:



and that "those organisms are capable of reducing CO_2 photosynthetically without the liberation of oxygen, because O_2 is the dehydrogenation product of the H-donor only in case the latter is H_2O (or H_2O_2)."¹ Van Niel and Müller (63) also venture an interesting hypothesis of the rôle of the yellow and red pigments in photosynthesis as activators of donor-hydrogen. Schneider (51) has also isolated the pigments from purple bacteria grown in pure culture.

THE FIRST SUGAR OF PHOTOSYNTHESIS

For a long time it has been the generally accepted view that the final product of photosynthesis in the form of carbon compounds is primarily some carbohydrate. There is, however, no unanimity of opinion concerning the exact nature of the first sugar formed in photosynthesis. The simplest chemical conception of the process would suggest that the first product is a hexose sugar which is converted into other hexoses and condensed to disaccharides such as sucrose and to polysaccharides as starch. But when this conception has been tested by chemical analysis of the different sugars present in green leaves, the results have not been unequivocally in its favor. There are a number of difficulties which are encountered in this approach to the problem. In the first place, the various carbohydrates are subject to interconversion, involving the change of one hexose into another, condensation of these into di- and polysaccharides and hydrolysis of these again into their simpler components. Thus we are dealing with an exceedingly complex "dynamic equilibrium," the changes in which are brought about by conditions which are as yet imperfectly understood. In the second place, the sugars in the leaf are continually subject to translocation and consumption by respiration. The amounts present at any one time thus represent a steady state which may be affected by a variety of factors. Finally, the exact analysis of the carbohydrate mixture in fresh leaf material is by no means a simple task; different types of leaves present different problems and the relatively large and varying amount of non-carbohydrate material present in all leaves makes satisfactory analytical procedure very difficult. It cannot be said that thoroughly satisfactory methods for this purpose have as yet been developed. The question is, therefore, whether the relative amount of any particular sugar present in the leaf, or the course of change in quantity of these sugars, really gives any indication of the first sugar which is formed.

Barton-Wright and Pratt (3) from the results of hourly analyses

of the sugars in the leaves of *Narcissus* conclude that the first sugar of photosynthesis is a hexose. They emphasize the correlation between the hexose and sucrose values and the fact that there is a lag between increase in hexose and increase in sucrose. This plant forms no starch, so that the two forms of carbohydrates mentioned were the only ones which need be considered.

Clements (7) has made analyses of carbohydrates in leaves and petioles gathered hourly and in one case at 10-minute intervals, of potato, soybean, and sunflower plants. These analyses show that sucrose is not more abundant than the reducing sugars, nor that its content varies more. On the basis of these analyses Clements concludes that the arguments which have been advanced in favor of the idea that sucrose is the first sugar synthesized are untenable and, further, that it is the simpler sugars (hexoses and pentoses) which are the first to be formed. The analyses also show great fluctuations in starch-content of the leaves as well as of the hemicelluloses. The latter are regarded as important food reserves and as being associated with drought resistance. The exact definition of the latter group of compounds has as yet not been definitely established and must await further chemical work on their constitution. Although sucrose also appears in the petioles, it is not as abundant as the simpler sugars and Clements concludes that the latter is the usual form in which carbohydrates move from one part of the plant to another. Clements (8) found no mannose in the leaves of the forty-two species he examined.

In this connection the investigations of Schroeder and Herrmann (53) are of interest. They have shown that in wilting nasturtium leaves, compared with perfectly fresh ones, the sucrose content increases at the expense of starch. The interconversions which some of the hexoses must undergo in this change are as yet not understood, but this change serves to illustrate the intricate reactions which are involved in the carbohydrate economy of leaves under conditions in which the water-content of the leaves decreases. What results minor variations in water-content of leaves bring about in the carbohydrate equilibrium requires further investigation.

PHOTOSYNTHESIS *in vitro* AND THE FORMALDEHYDE THEORY

The formaldehyde theory continues to arouse interest with the result that new contributions to the problem of the first reduction product of carbonic acid continue to appear. These embrace investi-

gations on systems *in vitro* as well as with living plants. In spite of all of this work we are apparently no nearer a unanimous opinion or to decisive judgment one way or the other. As examples of the great divergence of opinion which still exists concerning the reduction of carbonic acid to formaldehyde by means of light outside of the living cell the following opinions are quoted. The first is by Professor D. Vorländer, who developed the use of dimethylhydro-resorcinol (methon) as a delicate test for aldehydes, a test which has been extensively used in investigations on this subject. He states (65) :

Bis jetzt hat noch kein Sterblicher den nach der hypothetischen Assimilationsgleichung aus Kohlendioxyd, Wasser und Licht zu erwartenden Formaldehyd sicher und einwandfrei nachweisen können, weder bei Gegenwart, noch bei Abwesenheit organisierter Materie.

On the other hand we have the statement by Professor F. G. Donnan (14) :

The question arises as to how organic substances could have arisen by degrees in a primeval ocean originally containing only inorganic constituents? The late Professor Benjamin Moore took up this subject and endeavored to prove that colloidal iron oxide, in the presence of light, moisture, and carbon dioxide could produce formaldehyde, a substance from which sugar can be derived. This work of Moore's has been actively taken up and developed by Professor Baly in recent years. He has conclusively proved that, in the presence of light, moisture, and carbon dioxide, formaldehyde and sugar can be produced at the surface of certain coloured inorganic compounds, such as nickel carbonate. We may therefore conclude that the production of the necessary organic substances in the primeval ocean offers no insuperable obstacle to science.

Baly has previously reported the synthesis of organic compounds by the action of visible light on the finely divided carbonates of nickel and cobalt suspended in a saturated solution of carbonic acid. From the experience of Baly and his co-workers, as well as that of other workers, the preparation of these carbonates in a form which assures photosynthesis is associated with considerable difficulty. Baly (1) emphasizes that the preparations must be free from alkali and states that their partial solubility in carbonic acid, their decreased activity when kept in a dry state, and the loss of this activity under the influence of light constitute definite disadvantages. Recently he has described preparations of kieselguhr for which superiority is claimed over the former preparations. With kieselguhr, previously coated with aluminum hydroxide, and supporting nickel carbonate with a small amount of ThO_2 , a yield of organic matter photosynthesized in two hours at 25° of 0.0025 gram per gram of powder is reported.

The most promising results were obtained from aluminated kieselguhr preparations supporting ferric oxide and thorium oxide. Baly lays great stress on the importance of the surface potential of these preparations. He says:

As a result of numerous experiments it has been proved that no photosynthesis is obtained with a powder the particles of which carry an electronegative charge. Furthermore, the application of cataphoresis measurements to preparations of nickel and cobalt carbonates has given an explanation of the difficulties we ourselves met with in employing them as catalysts and the negative results obtained by others in an attempt to reproduce our earlier observations.

The central problem in the preparation and rôle of these catalysts is still quite obscure. When Baly tried to produce the preparations with fresh samples of kieselguhr it was not possible to reproduce the results and, although it is reported that with these ferric oxide catalysts the "maxima and minima of photosynthesis occur at the maxima and minima of surface potential," he states, "the yields have at best been far smaller than our earlier work led us to expect, and at times have been vanishingly small."

Dhar and his co-workers (13, 44, 45, 46, 47) have published a number of papers on the synthesis of formaldehyde from carbonic acid in "tropical sunlight." In general their results confirm those obtained by Baly and his collaborators. Dhar reports having obtained tests for formaldehyde from carbon dioxide and water in the presence of photosensitizers and from alkali bicarbonates in the presence of photosensitizers. The reactions were carried out in pyrex and soft-glass vessels; marble and hydrochloric acid were used to form the CO₂, and Schryver's or Déniges' tests were used to detect the formaldehyde. The periods of insolation were from three to sixty hours. There is unfortunately no information concerning the intensities and spectral composition of the "tropical sunlight" which was used, beyond the statement: "But as ordinary glass transmits wavelengths only up to 3,400 Å, it can safely be asserted that not much ultra-violet light takes part in our experiments." Blank experiments in which conductivity water and CO₂, or a solution of the photosensitizer without CO₂, were exposed to sunlight are reported as yielding no formaldehyde, and this fact is utilized as effectively removing the possibility of impurities being responsible for the formation of formaldehyde. An extensive series of photosensitizers was used, consisting of colored inorganic salts and organic dyes. Some "semi-quantitative" data are given of the percentage of formaldehyde

formed in visible light. Thus, with CO_2 passed into conductivity water, the blank formed "not much" formaldehyde, CO_2 in manganous chloride solution, 0.0008 per cent formaldehyde by volume; in nickel carbonate, 0.00008; in copper sulphate, 0.000081; in cobalt carbonate, 0.0006; in copper acetate, 0.00004; in chromium oxide, 0.00006; in malachite green, 0.00006; in cobalt chloride, 0.00004; in nickel sulphate, 0.00004; in uranium nitrate, 0.00005. It is stated that "ultraviolet light increases the yield of formaldehyde." This increase is in the neighborhood of 0.000005 per cent. From solutions of sodium bicarbonate 0.0032–0.0034 per cent formaldehyde was reported.

Reminiscent of the idea put forward by Stoklasa and Zdobnický (57) in 1911 is the statement of Atma Ram and Dhar that, "with nascent carbon dioxide obtained by the interaction of carbonates and hydrochloric acid no colored photosensitizer appears to be necessary for the formation of formaldehyde." Thus with barium, strontium, and calcium carbonates to which hydrochloric was added during insolation about 0.0002 per cent of formaldehyde is reported as having been formed.

Although Rao and Dhar (46) report having obtained "appreciable quantities of formaldehyde and carbohydrate" from the insolation of a 2 per cent solution of sodium bicarbonate and nickel carbonate, Atma Ram and Dhar (44), using CO_2 or NaHCO_3 and various photosensitizers, report no positive tests for the presence of carbohydrates. They report that solutions of formaldehyde are "polymerised" into sugars in the presence of photosensitizers, notably ferric chloride. Dhar and Atma Ram (11) also report the reduction of carbonic acid, carbonates, and bicarbonates to formaldehyde by means of metallic magnesium or zinc, or ferrous carbonate in sunlight. They state that, contrary to the older observations of Fenton, no formic acid was detected. It should be noted that the reduction of formic acid to formaldehyde by means of magnesium is the basis of an old and well-known test for formic acid (48). Dhar and Atma Ram (12) also report the presence of formaldehyde in rain water.

A repetition of Baly's earlier experiments on the photosynthesis of carbohydrates from carbonic acid by means of visible light and solid colored catalysts, following these in detail as closely as possible, was undertaken by Zscheile (66). He has described with some particularity the method he employed for the preparation of the catalysts, especially the carbonates of nickel and cobalt. He also used Co_3O_4 deposited on pumice, charcoal from sucrose, the oxides of nickel

and cobalt, ferric oxide and ferric hydroxide, colloidal cupric oxide, and platinum. Special care was exercised to remove impurities from the carbon dioxide used in the experiments. The results were all absolutely negative. MacKinney (36), using NiCO_3 and $\text{Fe}(\text{OH})_3$ as catalysts, with insolation of 60 hours, obtained only negative results.

Emerson (15) also attempted to repeat Baly's experiments. In this case the experiments were designed to demonstrate manometrically and by gas analysis, in a closed system, the disappearance of carbon dioxide and the formation of oxygen. Emerson also made an experiment with a continuous stream of CO_2 . He could detect no disappearance of CO_2 , no formation of oxygen, nor any organic material.

Bell (4) has reported on experiments carefully designed to procure active catalysts. A series of kieselguhr-supported catalysts, containing from 1 to 26 per cent Fe_2O_3 and a small amount of ThO_2 , had an electro-positive charge when suspended in solutions of CO_2 . The nickel carbonate was prepared electrolytically and activated by illumination before using. In order to determine whether the carbonic acid had been reduced, tests were made for formaldehyde and for carbohydrates, and carbon estimation was made in the residues by a wet-combustion method. No evidence of any photosynthesis of carbon compounds was obtained. Qureshi and Mohammad (43) also report only negative results by the use of ultra-violet, sunlight, and tungsten-filament lamps and various inorganic and organic photosensitizers. They ascribe the positive test for formaldehyde reported by others to impurities in the chemicals used.

Dimethylhydroresorcinol (5,5-dimethyl-cyclohexandian-1,3), introduced by Vorländer as a delicate test for formaldehyde, has been extensively used both *in vitro* and *in vivo* in order to determine whether formaldehyde is formed from carbonic acid on illumination. This reagent has been commonly called "dimedon," contrary to the expressed wishes of Vorländer (64), who suggested the term "methon" for dimethylhydroresorcinol and "formal-dimethon" or "methylene-dimethon" for the formaldehyde compound. Although it falls outside of the period under review here, the fact may be recorded that Vorländer, using his reagent, was unable to establish the formation of any formaldehyde on illuminating solutions of CO_2 with ultra-violet light or with sunlight for months, both with and without a variety of salts.

Klein and Werner (28), using dimethylhydroresorcinol, had reported that formaldehyde was formed as an intermediate product of

photosynthesis in plants, especially in aquatics. The formaldehyde was supposed to have been detected in the nutrient solution as well as in the plants themselves. Barton-Wright and Pratt (2) made tests with this reagent for the formation of formaldehyde by *Elodea canadensis*. They conclude that in bicarbonate solutions, in the presence of light, appreciable amounts of formaldehyde are formed and that this process is independent of the photosynthetic mechanism of the plant; the results of Klein and Werner are therefore regarded as not establishing the formation of formaldehyde as an intermediate product in photosynthesis. Barton-Wright and Pratt unfortunately do not report on what would be the crucial experiment in their series, viz., the possible formation of formaldimethon independent of the presence of carbonic acid. An explanation of the results of Klein and Werner and of Barton-Wright and Pratt is probably to be found in the property of the methon, which is itself capable of being oxidized to formaldehyde, the latter then reacting with unchanged methon with the formation of formaldimethon. This possibility of obtaining spurious results had been pointed out by Vorländer (65), who showed that methon behaves in this manner and is therefore not a suitable reagent for formaldehyde under conditions where it is apt to undergo oxidation.

The formaldehyde theory is indeed in a singular state. For over twenty years many investigators have attempted to achieve a reduction of carbonic acid to formaldehyde or to carbohydrates by means of light without the living organism. With the positive results, completely negative ones appear synchronously. These experiments are, it would seem, relatively simple. So wherein lies the cause for this complete lack of concordance? It is difficult to avoid the conclusion that the burden of proof rests with those who maintain that positive results have been attained. Certainly one of the corner stones of our scientific system of thought is reproducibility of results and this in turn rests upon the complete, accurate, and unambiguous description of all conditions pertaining to the phenomena observed. Without this, knowledge would become occult and mystical.

For over sixty years the formaldehyde theory of photosynthesis has allured chemists and plant physiologists. To a large measure this has doubtless been due to its fascinating simplicity, to the authoritative position enjoyed by its proponent, Adolf von Baeyer, and to the absence of an equally feasible idea. In total, the work done on the testing of this theory represents an enormous effort; in all of science

there are probably few ideas of equal significance which have survived so long without having been established or definitely discarded. Even to those who have yielded to its lure it has not served as a step to a deeper and clearer insight into the process as it occurs in the plant.

PHOTOSYNTHESIS UNDER NATURAL CONDITIONS

Increasing attention is being given investigations of photosynthesis under natural or field conditions, and, while these are primarily of ecological interest, some of the results are not without value in their bearing on the mechanism of the reaction, especially in connection with experimentation with living plants. Limitation of space permits but the briefest mention of these investigations.

Some of the complexities associated with the endeavor to subject photosynthesis to more rigorous experimental study are brought to light when the rate is followed under constant external conditions, i.e., of temperature, light-intensity, and CO₂ concentration. Harder (21, 22) found that under such conditions the rate was by no means constant. *Fontinalis* and *Cladophora*, kept in the dark for many hours, on illumination gradually increased in rate for several hours. When the illumination was interrupted for 20 to 30 minutes the same rise continued. Only when the periods of darkness were long (6 hours) was there found a subsequent lower rate on illumination. After long periods of illumination there again occurred a decrease. The latter was deferred by interspersing the period of illumination with short periods of darkness. The importance of obtaining data of this nature, toward which Harder has made a valuable beginning, is great, especially in emphasizing the significance of considering the recent history of plants used in experimentation.

A number of investigations on the ecological aspect of photosynthesis have been published by the late Professor Kostytschew and collaborators. They had (29, 31) found that the higher values of photosynthetic activity which were obtained by means of Sachs's half-leaf method over those found by the direct method of CO₂ determination were due to a deficiency in CO₂ supply in the latter method. With a more rapid air stream (30-60 liters per hour) approximately the same values were obtained with the two methods, and Kostytschew, Bazyrina, and Tschesnokov (30) devised a field method on this basis which was used for a series of investigations of the rate of photosynthesis under natural conditions. Kostytschew maintained that the rate of photosynthesis during the course of the

day is very irregular, and that this irregularity is probably not directly associated with changes in external conditions; that the rate can be very high in atmospheric air, and that the effect of increasing the CO_2 concentration is probably an indirect one. The concentration of CO_2 and air temperature are thus regarded as not constituting limiting factors in the usually accepted sense; changes in these factors exert their influence through a chain of stimulatory reactions. On the other hand, the intensity of light may, under circumstances, be a limiting factor, although it also results in a complex of stimulatory reactions. These conclusions are based upon investigations carried out under a wide range of natural conditions (52, 53, 54). Tschesnokov and Bazyrina (58), in a theoretical paper, point out how a change in any one of the external factors results in a whole series of internal changes which in turn may influence the rate. They maintain that it is impossible to differentiate the effect of a single external factor as directly influencing the rate; the only proper method of investigating the influence of external factors is through the determination of the directly limiting factors. Such factors are primarily internal ones and only after thorough investigations have been made of these factors within the plant can generalizations be drawn concerning the influence of purely environmental conditions.

The investigations of Müller (39) reveal some interesting relations of light and temperature in photosynthesis during the Arctic summer. Harder, Filzer, and Lorenz (23) have made an extensive investigation of photosynthetic activity under the extreme conditions of aridity, temperature, and light-intensity existing in the Sahara Desert, showing that the highly specialized organisms of this region do photosynthetic work even under the severest conditions, e.g., 50° C. Schoder (52) has shown the great variability of photosynthetic activity in relation to external factors in plants growing under natural conditions. This is also apparent from the results of Hiramatsu (24). The conditions resulting in the high production of organic material in the tropical rain forest, notably temperature, have been studied by Stocker (56) and some observations on the light conditions have been made by Mortensen (38). In this connection the findings of Jaccard and Jaag (26) relative to the variations of CO_2 absorption during continuous illumination are of significance. Boysen-Jensen (6) has summarized the results of his extensive investigations on the production of organic material by plants, especially in forests, in which the important environmental and edaphic factors are con-

sidered from the point of view of their influence on photosynthesis. Boysen-Jensen and Müller could not confirm the results of Kostytschew and his collaborators concerning the great fluctuations in photosynthetic rates under natural conditions. The work is an important contribution to the introduction of scientific methods into ecology.

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MINERAL NUTRITION OF PLANTS*

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In preparing this chapter, the same policy was followed as last year. No attempt was made to cite every article consulted by the reviewer. It is planned that in the next volume another reviewer shall assume the task of writing the chapter and thus a new point of view be presented. No doubt certain topics inadequately treated in the first two volumes will receive proper consideration in later volumes. For the benefit of new readers, it should be stated once more that it is not the intention to deal with soil and fertilizer investigations, save incidentally.

Chemical elements essential for plant growth.—McHargue & Calfee (24) investigated the lettuce plant with reference to its boron requirement. They demonstrated that this element is indispensable for normal growth and that the function of boron cannot be performed by any other element tested, including manganese, copper, zinc, nickel, cobalt, barium, strontium, iodine, and arsenic. The same investigators have also developed a spectroscopic method for the determination of minute amounts of boron present in plant tissues (23). Very little information is available on the distribution of boron in different plant tissues, and it is to be hoped that such improvements in analytical methods will lead to detailed studies of this kind.

Although minute quantities of boron are essential for plant growth, this element easily becomes toxic at very low concentrations in the nutrient medium. Under certain conditions, injury caused by boron assumes practical importance. This is true when certain types of irrigation water containing boron are employed. Scofield & Wilcox (40) reported extensive results on the boron content of irrigation waters and have included in their report data of physiological interest on the nature of boron injury to plants. Two especially susceptible crops are the lemon and the Persian walnut. Morris (26) studied the toxicity of boron for wheat plants under controlled conditions. Fifty parts per million of boron in the nutrient solution

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caused 40 per cent retardation of growth. Other investigators have found that very much smaller concentrations than this are highly injurious to some plants.

In the previous review, reference was made to the fact that the essentiality of manganese for the growth of *Lemna major* had been questioned. McHargue & Calfee (25) re-investigated this plant with reference to its manganese requirements and secured definite evidence that lack of manganese results in retarded growth, failure to reproduce, chlorosis, necrosis, and death after utilization of the residual metal in the plants. It becomes very dubious, therefore, whether there is any evidence that manganese is dispensable for any chlorophyll-bearing plant. Haas (16) studied the effects of iron and manganese deficiencies on citrus. Manganese was found to be essential and not replaceable by iron. Numerous illustrations are presented showing in detail the nature of leaf symptoms associated with iron and manganese deficiencies.

Investigations conducted during recent years have proved that zinc is essential to the growth of some, if not all, higher plants. Roberg (37) reported the results of an investigation of the effects of zinc on the growth of several forms of algae. He was unable to demonstrate that zinc is absolutely essential to the growth of the organisms studied, although he employed doubly distilled water and carefully purified salts. However, he did secure what he designates as stimulative effects. Porges (34) observed very marked effects of zinc sulphate on the metabolism of *Aspergillus niger*, including more rapid utilization of sugar and increased production of citric acid. Lignin-like substances were decreased over 50 per cent by the treatment.

A new and apparently economically important effect of zinc on the growth of fruit trees is described by Chandler, Hoagland & Hibbard (9). It was discovered that the non-parasitic disease known as "little-leaf" could be cured in most of the cases observed by the application of zinc salts to the soil, or by applying such salts directly to the tree through holes bored in the trunk. It is doubted whether zinc is required in the amounts used for normal metabolism. Certain experimental evidence indicates that, under the soil conditions involved, certain fluorescent bacteria of the soil may produce highly toxic compounds, which in some way may be rendered innocuous through reaction with zinc. Proof of this theory is still incomplete. It is interesting to note that an independent investigation of Alben &

Cole (2) in another section of the United States disclosed the fact that zinc sprays have a curative influence on the disease of pecan rosette. Anderssen, working in South Africa, showed that copper salts cure a disease affecting fruit trees growing in certain soils (3). Highly chlorotic leaves were made green by immersing them for a period in very dilute copper-sulphate solutions. In this case, also, the evidence is apparently not conclusive that mere deficiency of copper for normal metabolism is the cause of the disease. Nevertheless, such experiments as those just mentioned raise certain new and important questions pertaining to soil and plant interrelations. Each year, it becomes more evident that the study of the mineral nutrition of plants must take into account chemical elements previously neglected.

Availability of iron and plant growth.—During the past twenty years, the literature of plant physiology has contained considerable discussion of the question whether higher plants require or are stimulated in their growth by organic accessory food substances of vitamin-like character, derived from the soil. Certain investigators have believed that the results of experiments warranted an affirmative answer to this question and the name "auximones" was sometimes applied to such supposedly stimulative organic compounds. Other investigators advanced the view that the stimulating effects observed were not caused by any organic substance but rather by iron impurities contained in the organic preparations employed in the experiments. However, the evidence presented seems not to have been conclusive and the question has remained to some extent undecided. Burk, Lineweaver & Horner (7, 8) published during the past year details of a very thorough and critical investigation of the influence of "humus" on the growth of *Azotobacter vinelandii*. Various independent lines of evidence are adduced to show that humic acid causes stimulation of growth primarily by virtue of its iron content; the organic fraction is substantially inactive. A statistical study of extensive quantitative data showed that a "given suboptimal amount of soluble iron gives the same order of magnitude of increased growth, independently of whether the iron is added as contained in natural humic acid or in any of its substitution compounds." Although the data pertain strictly only to Azotobacter, it is suggested that the main conclusions may have a more widespread application to the mineral nutrition of plants in general. These researches may throw further light on certain functions of organic matter in soils.

It is also possible that the humus compounds described may have a practical application in connection with water- and sand-culture experiments.

The relation of iron to chlorosis has been the subject of manifold investigations, yet the internal state of iron in the plant is still very incompletely understood. Plants chlorotic from lack of available iron may sometimes contain as much, or more, total iron per unit weight of tissue than do normal plants. Rogers & Shive (38) have considered further the factors affecting the distribution of iron in plants. Microchemical and macrochemical methods were utilized. The influence of internal pH on solubility of iron is emphasized. Plants which yield composite tissue fluids of low pH in general show relatively high soluble iron and low total iron. On the other hand, Oserkowsky, working with the tracheal sap extracted from the branches of green and chlorotic pear trees, did not find clear evidence that the pH regulates the concentration of iron in the sap, at least when this concentration is very low (28). However, it is not certain that the pH of the extracted tracheal sap represents that of the sap as it exists in the plant. Another study of chlorosis as influenced by iron and manganese was described by Chapman (10).

Absorption and accumulation of mineral elements by plant cells.—In the last report brief mention was made of the experimental work of Steward on potato tissue. Several articles have been published during the past year and further discussion of the general conclusions presented is desirable, since they are significant to a fundamental aspect of the mineral nutrition of plants. It may be recalled that the problem is to determine the relation of the metabolic activities of the cell to the accumulation of certain elements in the cell-sap. As in the case of earlier experiments on Nitella cells, bromides were found to be very suitable for studying accumulation, since bromine ions were not toxic and were not originally present in the tissues. When the potato tissues (which were cut into thin disks of accurately controlled thickness and subjected to rigorously controlled experimental conditions) were merely immersed in dilute bromide solutions, no accumulation of bromine in the sap occurred; that is, the concentration did not rise above that of the outside solution and in fact was generally lower. However, by passing a stream of air through the solution, the tissues were induced to accumulate bromine (and potassium also) with the result that concentrations within the cell rose to far higher levels than were present in the culture medium.

from which the elements were absorbed. It is concluded that the accumulated salt existed in true solution within the sap. Conductivity data favor this assumption. A very definite correlation is found between aerobic respiration and accumulation of potassium and bromine. Accumulation ceases at temperatures below 5° to 6° C., and over certain ranges the process has a high temperature coefficient, not characteristic of diffusion processes.

One series of experiments on potato tissue was made for the purpose of determining the relation of disk thickness to respiration and accumulation. Surface cells respire at a high rate and those innermost at a very much lower rate. The variables which affect respiration per unit weight of tissue also affect in a similar manner the salt absorption per unit volume, as represented by the internal sap concentration of the disks. Apparently all the absorbed bromine resides in the surface cells whose respiration is augmented and which exhibit certain cytological changes. "Salt absorption in potato thus appears to be a property, rather of cells embarking upon a new cycle of intense metabolism, which may even culminate in cell-division, than of cells which are truly dormant."

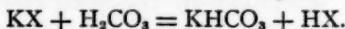
Many investigations of storage tissues are on record, but in nearly all cases the importance of aeration and of cell-metabolism has been overlooked or underestimated to such an extent as to lead to doubt whether significance can be attached to earlier conclusions, in so far as the nature of the process of accumulation by living cells is concerned. The significance of "absorption ratios" also must be reconsidered. If accumulation occurs only in surface cells, then comparison of the whole mass of tissue with the outside solution becomes of doubtful validity in formulating physical-chemical hypotheses concerning such ratios. Furthermore, any equilibria attained seem to be of dynamic character, rather than of the nature of adsorption equilibria. It may be added that other investigations [Hoagland & Broyer (17)] indicate that the absorption of mineral elements by root-cells is also dependent on metabolic activities, and that the process has a high temperature coefficient over certain ranges of temperature. Incidentally, it was shown with barley plants that metabolic activities of root-cells, rate of accumulation of electrolytes, and guttation were directly interrelated.

The mechanism possibly involved in accumulation, especially with reference to the Valonia cell system, continued to receive very active study by Osterhout and his associates. In the further develop-

ment of earlier theories, Osterhout & Stanley (30) have constructed a cell model which accumulates potassium. This model consists essentially of a non-aqueous layer, separating an acid aqueous layer, representing the cell-sap, from an alkaline aqueous layer, representing the external solution. The non-aqueous layer was composed of a guaiacol-*p*-cresol mixture. This system was found to be capable of accumulating potassium under certain conditions, chief of which was the maintenance of an acid reaction in the layer representing the sap. The principal means employed for maintaining the acidity of this layer was the passage of a stream of CO₂ gas. The operation of the system is best illustrated by citing the data given for a specific experiment. During the first 71 hours, no CO₂ was bubbled through the inner layer and potassium entered only until its concentration was equal to that of the external solution. The bubbling of CO₂ was then commenced and in the course of 312 hours the concentration of K in the inner layer rose until it was 12 times that of the external solution. From this time on, water was taken up as rapidly as electrolytes and the concentration in the inner layer remained approximately constant. In other words, a steady state was reached. The mechanism of penetration is further described as follows: KOH combines at the outer surface of the non-aqueous layer with an organic acid HX according to the reaction



At the inner surface another reaction takes place,



The net result is that the outer layer loses a potassium ion and the inner layer an H-ion. While this is thermodynamically equivalent to an exchange of K⁺ for H⁺, the mechanism is conceived to be very different from that advanced by other investigators whose theories are based on ionic exchange. [See also Jacques & Osterhout (18), Osterhout (29), Damon (11, 12).]

This model is of special interest because potassium is accumulated within the cell, attaining a higher concentration than exists in the outer medium, and because potassium is accumulated to a greater extent than sodium. Certain properties of the plant-cell are thus imitated, but, as the investigators themselves point out, the imitation is only partial. The artificial cell did not have the ability to accumulate chlorine as does the Valonia cell. Attention should also be directed to the point that in the theory described (and this applies

also to certain theories of ionic exchange) a gradient of H-ion concentration, or activity, between cell-sap and outer medium is essential to the operation of the mechanism. In the case of Valonia and Nitella cells growing under their natural conditions, such gradients between sap and medium do exist. However, the ability to accumulate potassium seems to be an almost universal characteristic of plant-cells, including root-cells, and the question arises whether hydrogen- or hydroxyl-ion gradients in the right direction and of suitable magnitude are always present. It is very difficult, perhaps impossible, to measure with exactitude the hydrogen-ion concentrations of the sap of individual cells making up a complex plant tissue, yet certain evidence suggests that conditions conforming to the requirements of the theories referred to above may be absent and potassium accumulation still occur. Specific data on this point obtained in the laboratory of the present reviewer raise doubt of this kind. It is, of course, conceivable that more than one type of mechanism may bring about accumulation of potassium, but it is also conceivable that a general and still-undiscovered mechanism exists.

With regard to carbonic acid as a means of maintaining an acid reaction in the cell-sap or of supplying HCO_3^- for exchange purposes, it might seem that the experiments of Steward (41-44) offer evidence that the respiratory activities of the cell furnish the necessary conditions, but it may be well to recall that Nitella cells can accumulate both K^+ and Br^- under conditions of continuous illumination, with the available CO_2 being removed from the system through photosynthetic action.

Reference was made last year to investigations on two closely related species of Halicystis, both living in sea-water, but one species (*H. ovalis*) accumulating potassium in the sap to a concentration much above that of sea-water and the other (*H. Osterhoutii*) having a sap not very different in composition from sea-water. Blinks (5) has continued this investigation and studied the potential differences across the protoplasm. The mean value for *H. Osterhoutii* was found to be 68.4 millivolts, that for *H. ovalis* 79.7 mv., the sea-water being positive to the sap. When the potassium content of the sap of *H. Osterhoutii* was experimentally raised so as to resemble that of *H. ovalis*, the potential was increased to a value about the same as that of the latter. Apparently the difference between the two species cannot be related to a difference in the mobility of K ions in the protoplasm.

Briggs (6) presented a theoretical treatment of the absorption of salts by plant tissues, considered as ionic interchange. It is suggested that "salt absorption by mature plant cells from weak solutions consists essentially of an exchange of anions between solution and cell-sap and of cations between solution and cytoplasm (and perhaps wall)." It has been shown by the early work of Devaux that such cation exchanges may occur, but it is not evident how the above-mentioned theory can explain the striking accumulation of potassium in the cell-sap of living cells of Nitella, Valonia, potato tissues, etc. The theory was elaborated before the results of Steward, already cited, became available.

Pirsche (33) described very extensive observations on the relative toxicity of different ions as measured by the length-growth of shoot and root of various agricultural plants. Although the complications of the system are recognized, Pirsche is led to emphasize the parallel between physiological effects and ionic radius in homologous series of ions. The effect of ionic hydration is considered to be important. Lundegårdh (22) has also considered this question in the monograph cited in the next paragraph. He points out that it is important to take into account the influence of ionic exchanges between plant roots and the culture medium in any attempt to relate ionic radius to physiological action. Ruhland, Ullrich & Yamaha (39) have likewise discussed this general question, on the basis of their experiments with *Beggiatoa mirabilis*. They believe that electrolytes may enter the cell as ion pairs (not undissociated molecules) with ions of similar size tending to form the pairs.

Lundegårdh has collected in monographic form (in German) the results of studies on the absorption of mineral elements by plants as carried on during a number of years in his laboratory in Stockholm. A special feature of this monograph is the description of methods of spectral analysis for cations and certain anions present in plant tissues. Among other topics dealt with at considerable length is that of the interrelations between calcium, magnesium, and potassium in absorption. One chapter is given to the relations between absorption, colloidal properties of the protoplasmic membrane, and physical-chemical properties of ions.

Plant buffer systems and absorption of mineral elements.—Many investigators in the field of plant physiology have held the view that it is essential that the sap of some, if not all, types of living plant-cells be maintained at a definite hydrogen-ion concentration, within

certain limits. The question of sap-buffering systems thus becomes of importance. The systems concerned are in many respects different from those described for animal fluids. Organic acids and their salts have a predominant rôle in plant cells over certain ranges of hydrogen-ion concentration. It has been a rather prevalent idea among plant physiologists, in considering the acid metabolism of plants, that special emphasis should be given to the base calcium, as being necessary to prevent the sap from becoming too acid and also, by precipitation, to protect the plant against toxic effects of such acids as oxalic. Dunne (14) made an investigation of these points, experimenting with plants, especially buckwheat, grown in controlled nutrient solutions, which permitted the supply of calcium and other elements to be varied as desired. The evidence obtained does not support the assumption that calcium necessarily has an indispensable function in regulating sap reaction. It seems to be possible for potassium to serve as well, and in some plants the latter ion may be more important from this point of view, since it can be absorbed much more rapidly than calcium or magnesium. The data from experiments in which calcium carbonate was present in excess in the medium indicate that oxalic acid can be formed in increased amounts in response to increased calcium absorption, rather than that a fixed amount of this acid is formed in metabolism, requiring calcium for precipitation. Furthermore, the evidence is not in favor of the view that oxalic acid has a specific toxic action on plant-cells. In any case, oxalic acid could be precipitated out by potassium as well as calcium. The indispensable function of the large amount of calcium required by certain types of plants may not have a specific relation to the regulation of reaction.

Thoday & Evans (46) studied the buffer systems of certain succulent plant tissues. In the young succulent stems of *Kleinia articulata*, the main features of the titration curve could be reproduced by solutions of malic acid containing a small amount of aluminum, and in the older stems by malic acid and calcium phosphate. Illustrating the difficulty of working with plant tissues Thoday & Evans (45) found that, in *Mesembranthemum*, soluble calcium and soluble oxalate are present in different cells. When the sap is extracted, mutual precipitation occurs. Complexities of this type are constantly met with in investigations of plant biochemistry, and make very difficult the interpretation of chemical data on plant tissues.

Nitrogen absorption by plants.—In the previous review, consider-

able space was given to a discussion of the intake of nitrate and ammonium nitrogen by plants. During the period covered by the present review, comparatively few papers in this field have been received. Kultzscher (19) extended the earlier German investigations of the relation between the reaction of the cell-sap and the storage of ammonia nitrogen by plants. Plants representing numerous species were examined with reference to sap-acidity and the ratio $\frac{\text{amid-N}}{\text{NH}_3\text{-N}}$. In general, this ratio was low when the pH was low and high when the pH was high. Plants with a pH above 5.0 are classified as "amid" plants, but intermediate classes are recognized. Experimental alterations of environment were also employed, by placing plants in the dark, and by feeding with asparagin, ammonium salts, and calcium nitrate. The effects on the plants were found to be in general accord with the theory.

Eckerson (15) has made further progress in studying the nitrate-reducing ability of plants. Altering the periods of illumination produced certain very striking effects on the reducing power of plant tissues: for example, when the Biloxi soybean was grown in an 8-hour day and 16-hour night the amount of reductase was only 1 to 5 per cent that in plants grown in full daylight in April. Phosphate and potassium seemed to be necessary for the formation of reductase, but the presence or absence of nitrate or starch appeared to have no effect on reductase activity.

Addoms & Mounce (1) studied the cranberry plant in the course of investigations on the utilization of different forms of nitrogen by plants. Calcium nitrate, ammonium sulphate, and glycine were compared under sand-culture conditions. Vigorous plants were produced with ammonium nitrogen at pH 6 and pH 8, and with glycine nitrogen at pH 4 and pH 6. The plants grown with ammonium nitrogen at pH 4 and with glycine at pH 8 were stunted and chlorotic. The glycine was believed to be absorbed as such. The article does not make clear to what extent the initial reactions of the culture solution were maintained in the sand culture, so that it does not appear to be proved that pH 8 actually maintained at absorbing root surfaces is a favorable reaction for the use of ammonium nitrogen. Tiedjens & Blake (49) reach the conclusion that the apple tree can utilize the ammonium form of nitrogen readily, provided a suitable pH (above 6.0) is present. [For discussion of nitrogen metabolism in storage organs, see Rahn (35).]

Potassium nutrition.—Penston (31) completed a study of the distribution of potassium in the potato plant as evidenced by microchemical methods. In agreement with previous views, the data show that potassium is present in considerable quantity in regions of special physiological activity. A further suggestion is obtained of the relation between potassium and protein metabolism in the close association of potassium and protein in nearly all tissues. A similarly consistent association was not observed in the case of starch.

Extending earlier studies on potassium, Richards reported the effects of potassium deficiency on the rate of respiration in successive leaves of barley plants grown in sand culture, with four levels of potassium supply. Statistical methods were applied as in the earlier investigations. The relation between respiration and potassium is very complex. It was found that respiration rate increased in general as the level of potassium concentration was lowered, but there was an optimum concentration below which the rate again decreased. At very low levels of potassium supply, the positive correlation with respiration rate is apparently connected with the very low carbohydrate concentration present in the leaf. When enough potassium is available to permit the formation of abundant carbohydrate, there is a negative correlation between respiration rate and amount of potassium supplied. In explaining this the suggestion is made that the high amino-acid content of potassium-deficient leaves may influence respiration. [Berkner & Schlimm (4) reported the results of an investigation on effects of potassium on strength of straw, and Leibrandt (20) on the influence of potassium on the growth-rhythm of beet plants.]

Sulphur deficiency.—The effects of sulphur deficiency on the metabolism of the tomato plant were investigated in considerable detail by Nightingale, Schermerhorn & Robbins (27). Some of the characteristics of the sulphur-deficient plants were similar to those shown by plants deficient in other elements, but the sulphur-deficient plants had, in addition, a remarkable capacity for stem elongation. Reduction of nitrates and oxidation of sugars was comparatively slow in such plants, and they contained extremely high amounts of sugar and more nitrate than plants grown in complete nutrient solution. The sulphur-deficient plants had a low content of S-H compounds and there was practically no active cambium. It is believed that cell-wall thickness in tomato plants is more intimately associated with carbohydrate content than with any other single factor of nutri-

tion and these high-carbohydrate sulphur-deficient plants had very thick cell walls and a relatively high proportion of fibers and lignified tissue.

Toxic effects of mineral elements on plants.—For many years, there has been much discussion of the toxic effect of aluminum under soil conditions, without any general agreement being reached. This question is of great interest because of its relation to soil acidity. Many investigators are of the opinion that hydrogen-ion concentration *per se* is generally insufficient to account for the unsuitability for plant growth of certain acid soils. The factors of inadequate calcium supply and of aluminum toxicity have been invoked from time to time. Ligon & Pierre (21) now report new studies on the toxicity of aluminum for corn, sorghum, and barley. Aluminum was definitely injurious when present in the culture solutions in concentrations as low as 1 p.p.m. With increasing concentrations, injury was progressively increased. According to Ligon & Pierre, aluminum may be injurious to plants under acid soil conditions, since concentrations of aluminum similar to those producing injury in artificial culture solutions may be found in soil solutions of soils of pH 4.5 to 5.0 [Pierre, Pohlman & McIlvaine (32)]. Trénel & Frey (51) have also investigated the toxic effects of aluminum on plant growth, using the method of sand culture. Aluminum depressed strongly the absorption of calcium and magnesium, while the absorption of potassium was but little influenced.

Trelease & Trelease (50) have given a detailed description of effects of magnesium injury to wheat. Specific deformation of leaves was noted, beginning with the third leaf. The characteristic abnormality of the affected leaf was a spiral deformation within the sheaf, developing below the tightly rolled apical portion. The leaves were darker green than those of normal plants, numerous tillers developed prematurely, and growth of seminal roots was inhibited. It is concluded that the severity of injury is related to the ratio of magnesium to calcium in the culture solution. This work recalls an old question upon which there existed earlier much discussion, namely, whether certain specific ratios of available calcium to magnesium in the soil favor plant growth. No wide agreement was ever reached to the effect that such ratios were of physiological significance. However, the solutions of Trelease & Trelease causing magnesium injury had relatively high ratios of magnesium to calcium, which presumably do not often obtain in the case of soil solutions.

Review articles.—Thomas (47, 48) contributes two articles in which are contained extensive summaries of literature pertaining to the utilization of mineral elements by plants, especially with reference to soil and plant interrelations.

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THE CHEMISTRY OF BACTERIA*

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MULTIPLICATION

The subject of bacterial multiplication has begun once more to attract investigation. According to Regnier, David & Kaplan the method of the "viable count" may be responsible for serious errors. These workers, using *Pseudomonas pyocyannea* on a liquid medium, carried out a direct microscopic ("total") count and a "viable" count by the usual method of diluting, plating, and counting colonies. By the former method no latent period was observed, but the multiplication started immediately into the logarithmic phase. The viable count, however, disclosed the usual latent period; that is to say, no multiplication occurred for two hours. This seems to show that the latent period is an experimental artifact due to the transfer of cells from the liquid to the solid medium, and does not occur in the parent culture. Like Wilson (1922), the present authors found that, even after the latent period, when both methods of enumeration were giving a logarithmic rate of multiplication, the total count exceeded the viable. Hence it seems that in a growing culture a certain number of cells are capable of division whilst they remain in the liquid medium, but fail to divide when transferred to agar plates, and that this difference is so marked in the stages closely following inoculation as to give rise to a deceptive appearance of latency which does not actually occur.

A second and highly interesting phenomenon disclosed by the same investigators is the influence of crowding on multiplication rate (Regnier & Kaplan). Comparing the rates of multiplication with large and small sowings, it was found that the duration of the logarithmic period was the same in both cases, as was also the maximal number of cells produced; that is to say that bacteria sown in large numbers multiply at a constant rate, but more slowly than those sown in small numbers. This difference is apparent from the beginning and hence cannot be attributed to exhaustion of food ma-

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terial or to the accumulation of toxic waste-products; the mere crowding of cells appears to prolong the generation time. A similar effect was noted in 1892 in the case of yeast by A. Brown, who found that if cells were sown into fresh wort in numbers equal to or exceeding 28×10^7 cells per cubic centimeter, fermentation occurred without any cell-division. It seems then that micro-organisms are not so individual as at first appears, but that in close association they exercise some mutual effect on each other's rate of multiplication.

More evidence on this point is supplied by the ingenious experiments of Rogers & Whittier. These workers grew *Str. lactis* in an apparatus constructed so that the medium was constantly changed at a rate of from 1 to 5 times in 24 hours; the exit tube was fitted with a filter so that no outflow of cells could occur. In spite of the abundant supply of medium and removal of waste products, the population remained at a steady level of live cells (2.43 to 3.68×10^8 cells per cc.) for 7 days. The population level differed with different species, *Esch. coli*, for example (in slightly different conditions), maintaining a population of about 2×10^9 .

Other contributions of interest in this subject are: An investigation of the total crop of bacteria obtained on laboratory media of different composition and concentrations (Regnier & David); the preparation of an active concentrate from meat extract, such that 1 mg. added to 50 litres of synthetic medium supports the growth of *staphylococci* (Hughes); the proof that filtrates from tryptic broth cultures of *Bact. coli* when added to fresh broth do not inhibit the growth of fresh cells to a greater degree than does an equal volume of water (Barnes).

An extended study has been made on the effect of sodium chloride on the growth of *Bact. coli* (Winslow, Walker & Sutermeister). It was found that 0.1 M sodium chloride has a mildly stimulating and 0.5 M a mildly inhibitory effect when taken over the whole growth period. Higher concentrations were definitely toxic. In the lag period both 0.1 M and 0.5 M concentrations had an inhibitory effect and *both* had an accelerating effect in the logarithmic phase, as shown by the generation times.

Medium	Generation Time
Peptone water alone.....	36 minutes
Peptone water + 0.1 M NaCl.....	21 minutes
Peptone water + 0.5 M NaCl.....	16 minutes

A valuable study of metabolic activity as measured in carbon dioxide and ammonia eliminated per cell in various phases of growth has been made from the same laboratory (Walker & Winslow). Many interesting facts come out of this study. In the period of stable population on a 1 per cent peptone medium each cell eliminates 1.3×10^{-11} milligram of carbon dioxide and 0.2×10^{-11} milligram of ammonia per hour; this is approximately 2 mols. of carbon dioxide to 1 mol. of ammonia nitrogen. In a medium with 0.5 per cent peptone and 0.5 per cent lactose the carbon dioxide production is approximately the same (1.5×10^{-11} mg. per cell per hour) but the nitrogen is now less than 0.1×10^{-11} ; moreover the rate of multiplication and total carbon dioxide production are the same in the presence or absence of lactose, whilst the reduction of nitrogen appears from the start. This precludes the possibility that the ammonia is being fixed by increase of growth and points to a "sparing" effect of carbohydrate on "protein" decomposition, the CO_2/N_2 ratio rising in the lactose-peptone medium, 10 to 26 times its value in plain peptone.

Very remarkable also is the variation in the metabolic rate at various phases of the growth period; this is shown in the following table:

TABLE I

Medium	Production in mg. $\times 10^{-11}$ per cell per hour					
	CO ₂			NH ₃ · N		
	Lag	Logarithmic Increase	Peak Stability	Lag	Logarithmic Increase	Peak Stability
Peptone	42-99	52	1.3	10-24	4.5	0.2
Peptone+0.1 M NaCl	68-185	36	...	13-36	4.1	...
Lactose+peptone	41-104	54	1.5	6-16	0.5	<0.1

Toward the end of the lag phase in any medium there appears to be an enormous increase in metabolic activity, the carbon dioxide per cell per hour increasing 30 to 70 fold and the nitrogen 50 to 150 fold as compared with peak stability rate. A high oxygen consumption per cell has also been reported at the end of the lag period (Martin), but here the increase as compared with the peak stability is of a much lower order, and is correlated with an increase in the actual size of the cell of the same order of magnitude. Walker & Winslow attribute

these results to the high activity of "young" cells. Other factors, however, seem to need consideration. First, in the light of the work of Regnier, David & Kaplan with *Ps. pyocyanea* it seems possible that the viable count in this phase may be seriously low and information by means of the total count is needed. Secondly, the condition of the medium rather than the "age" of the cells may influence the rate of enzyme action; for instance, by the time the logarithmic phase is over, the concentration of some of the components of the substrate may have fallen below that necessary to saturate their respective enzymes, so that the observed falling off in activity of "old" cells may in reality be due to decreased concentration of substrate on the enzymes concerned. An examination into the viability of preparations of "resting bacteria" (*Bact. coli*) during the progress of dehydrogenation experiments with methylene blue (Sandiford & Wooldridge) shows that, whilst the total number of organisms remains approximately constant, the viable cells decrease. Notwithstanding this, the dehydrogenating power of the culture remains constant for about six hours. Thus during six hours' anaerobic incubation in the presence of lactate the viable count fell from 17×10^7 to 29×10^4 , whilst the velocity of reduction of methylene blue by lactate remained constant. During the average duration of a reduction experiment—say 20 minutes—the viable count fell from 5,453,000 to 471,500.

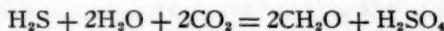
These experiments agree with earlier observations where aerobic oxidations were in question (Cook & Stephenson), and show that oxidative mechanisms are not correlated with viability.

THE COLOURED SULPHUR BACTERIA

We are now in possession of a study of the first importance on the purple and green sulphur bacteria (van Niel). It appears that these are of universal distribution in earth and mud, and in both salt and fresh water, and may be cultivated from these sources if the right conditions are complied with. These are:

- a) Exposure to light, either sunlight or from an arc lamp.
- b) A pure synthetic medium containing the following: NH_4Cl , 0.1 per cent; K_2HPO_4 , 0.05 per cent; MgCl_2 , 0.02 per cent; NaHCO_3 , 0.1 per cent; $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$, 0.1 per cent.
- c) pH about 8.5.
- d) Strictly anaerobic conditions.

By enrichment cultures followed by plating, 15 strains of purple and 4 of green sulphur bacteria were obtained. These were separated into groups according to the value of pH and concentration of sulphide at which they develop best. The metabolism of these organisms is now clearly established for the first time and is unique in nature. The purple bacteria are pure facultative autotrophants, and are strictly anaerobic. Sulphide (replaceable by sulphur, sulphite, or thiosulphate) is oxidised to sulphate, carbon dioxide being correspondingly reduced. Thus:



From estimations of sulphide used, sulphate formed, and carbon dioxide disappeared with a number of different strains, it is clear that the reaction takes place in accordance with the foregoing equation, as is seen from the following figures:

TABLE II

Number	Culture		H_2S Oxidised	H_2SO_4 Produced		CO_2 Disappeared	
	Description	Duration in Days		Calculated	Found	Calculated	Found
4	Stores sulphur inside of cell...	29	9.14	26.32	25.90	23.63	22.8
9	Stores sulphur inside of cell...	27	8.8	25.5	24.7	22.9	20.7
		42	18.7	53.9	51.4	48.4	46.8
1	Does not store sulphur	24	24.8	71.5	70.9	64.2	63.8
7	Does not store sulphur	32	11.39	32.83	34.2	29.48	28.5
		36	13.43	38.71	13.72	34.76	17.5

With strain 4, the H_2SO_4 produced is equivalent to 264 mols., which, in accordance with the equation, would require the reduction of 528 mols. of CO_2 , whereas 505 had disappeared. Moreover, after the sulphide had disappeared no further reduction of carbon dioxide occurred, showing the dependence of the latter process on the former.

The experiments with strain 9 show that after 34 days the sulphide had not been completely oxidised, and microscopic examination showed that the cells still contained sulphur globules; correspondingly the carbon dioxide disappearance was below the calculated

value. By 42 days the intracellular sulphur had disappeared and the sulphate formation then corresponded with the amounts of sulphide and carbon dioxide which had disappeared.

In culture 7 (second experiment) the sulphate formed was also low as compared with the sulphide used. Here this discrepancy was accounted for by an extracellular deposit of sulphur according to the equation $2\text{H}_2\text{S} + \text{CO}_2 = 2\text{S} + \text{H}_2\text{O} + \text{CH}_2\text{O}$. Thus out of 395 mols. of H_2S , 255 have been oxidised only to S, corresponding to the reduction of 128 mols. of CO_2 , so that the total maximum reduction of CO_2 would be $128 + (2 \times 140) = 408$ mols., of which 398 were verified experimentally.

In the case of the green bacteria, sulphide is oxidised only to sulphur which is deposited outside the cell. The disappearance of CO_2 corresponds with the equation



The photosynthetic character of the foregoing reactions is deduced from the fact that no oxidation of sulphide, nor development of bacteria (in the conditions described), occur in the absence of light. Moreover if the process were chemosynthetic rather than photosynthetic, one would expect, on the analogy of other chemosynthetic processes, that the sulphide oxidised would be largely in excess of the carbon dioxide reduced, a 5 to 10 per cent efficiency being the usual order obtained. Here, on the contrary, the carbon dioxide synthesized into cell material balances the sulphide oxidised, as in the case of the oxidation of water in the photosynthetic action of the green plant.

The general distribution of these groups has already been mentioned. It is only under certain conditions, however, that they develop richly, their requirements being exposure to sunlight and a supply of hydrogen sulphide with its attendant anaerobiosis. The source of hydrogen sulphide may be biological—decomposition of proteins or reduction of sulphate—or from sulphur springs. Deprived of these conditions they can carry on a heterotrophic life anaerobically on ordinary media, none of the strains examined being an obligate autotrophant. Radiant energy appears to be as necessary for heterotrophic as for autotrophic development, no growth in either circumstances having been obtained in the dark.

The mechanism of the photosynthetic reduction of carbon dioxide by the purple and green bacteria is still quite obscure. The subject

has been opened up from the theoretical standpoint by van Niel and Müller, and awaits experimental investigation.

NITRIFICATION

A new type of autotrophic nitrite-former has been isolated from the pinewood soils of North Carolina (Campbell). This organism (*Nitroso-bacillus, thermophilus Campbell*) is a strict thermophil whose optimum temperature is 55° to 60°, minimum temperature 40°. It withstood 100° for 8 hours and 120° for 45 minutes. It is facultatively heterotrophic.

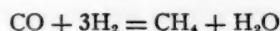
NITROGEN FIXATION

Winogradsky has applied a new technique to demonstrate the formation of ammonia as the first product of nitrogen fixation. Using silica jelly or agar plates and supplying carbon in the form of sodium lactate or succinate, which substances served also as sources of hydrogen for reduction, he found that in 24 to 40 hours the reaction of the medium corresponded to pH 9.0. Concurrently a marked evolution of ammonia occurred, which was estimatable by Nessler's reagent both in the gel and in the condensation water. No such evolution of ammonia took place in mannitol or glucose media, which became acid, nor in calcium lactate or succinate with excess of chalk, where the pH was fixed at about 8.0. Ammonia could, however, be demonstrated in plates of the last-mentioned medium if, after growth was attained, the plates were exposed to chloroform or toluene vapour. Winogradsky considers that the accumulation of ammonia at alkaline reactions and when the culture is exposed to vapours of anaesthetics is due to the inhibition of growth whilst the primary reduction of nitrogen is still proceeding.

The part played by iron in the stimulation of the growth of *azotobacter* by humin¹ has now been worked out in detail [Burk, Line-weaver & Horner (1)]. Molybdenum (as 0.0005 per cent Na₂MoO₄) has been found to have a definitely stimulating effect on nitrogen fixation (Bortels; Birch-Hirschfeld).

METHANE FERMENTATION

Continuing their researches on the production of methane according to the reaction



¹ Cf. *Ann. Rev. Biochem.*, 1, 649 (1932).

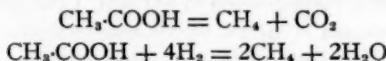
Fischer, Lieske & Winzer (2) have now shown that if CO/H_2 is greater than $\frac{1}{3}$, both CO_2 and CH_4 result, but if CO/H_2 is less than or equals $\frac{1}{3}$, CH_4 only results. The authors believe that, in the production of methane from carbon monoxide according to the equation above, carbon dioxide is an intermediate product, some evidence having been given in their previous paper (1) for the occurrence of the reaction



The reduction of carbon dioxide by hydrogen to form methane by mud cultures is now complicated by the appearance of acetic acid. In the presence of colloidal iron sulphide two reactions seem to occur:

- a) $\text{CO}_2 + 4\text{H}_2 = \text{CH}_4 + 2\text{H}_2\text{O}$
- b) $2\text{CO}_2 + 4\text{H}_2 = \text{CH}_3\text{COOH} + 2\text{H}_2\text{O}$

In the absence of colloids, b only occurs. The further fermentation of acetic acid with and without hydrogen gives methane:



It must be remembered that all these reactions occur in mixed culture, hence it is difficult to make valid deductions as to the course of a reaction, since alteration of conditions (e.g., presence or absence of colloids) may encourage the preponderance of different organisms.

FILTERABLE PROTEOLYTIC ENZYMES

It is now certain that the production of filterable proteolytic enzymes occurs not only on media containing proteins or their decomposition products, as was for long asserted, but can occur on simple amino acids and also where the nitrogen is supplied by ammonium salts and the carbon by simple known compounds—sugars, organic acids, or glycerol [Merrill & Mansfield Clark; E. D. Wilson; Haines (1, 2); Virtanen & Tarnanen (1)]. The part played by inorganic salts in the production of the exoenzymes is important and not yet clear. Merrill & Mansfield Clark, using a strain of *Proteus*, found that, whereas good growth was obtained on a medium containing sodium and potassium phosphate, ammonium chloride, and glucose (or lactate), no filterable enzyme was produced unless calcium and magnesium also were present. Wilson, using *Pseudomonas pyocyannea* and checking gelatinase production against bacterial counts, found

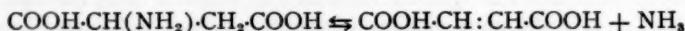
that the influence of the calcium and magnesium salts was attributable to their effect on growth; that is, the maximum bacterial population attained on a synthetic medium in the absence of calcium and magnesium was too small to admit of the filterable enzyme being obtained in demonstrable quantities. "If the maximum bacterial numbers observed in cultures in synthetic medium are compared with the number . . . in the same medium containing calcium and magnesium at an early stage in growth when not more than a trace of protease is demonstrable . . . the bacterial population of the one is comparable with that of the other."

Haines (1) and (2) advanced the subject in two directions. He employed a viscometer for measuring the effect of gelatinase on gelatin with gain in precision and in the measurement of low degrees of activity. Working, like Merrill & Mansfield Clark, with a strain of *Proteus*, he disagreed with Wilson (working with *Ps. pyocyanea*) in attributing the effect of calcium and magnesium entirely to growth. He also demonstrated that these two metals play distinct parts, the former stimulating growth but slightly inhibiting gelatinase production, the latter having little or no effect on growth but actively stimulating the formation of gelatinase. On a purely synthetic medium with both salts he obtained a gelatinase activity comparable with that given on a meat-broth medium. He also demonstrated that the effect of magnesium occurred during the growth of the organism and was not obtained if the salt was added to the filtrate after removal of the cells. In other words, its effect is during the production of the enzyme and is not a case of activation after the enzyme is formed.

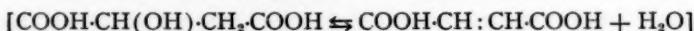
Working with *B. fluorescens liquefaciens*, Virtanen & Tarnanen note that the filterable protease when obtained on a broth- or milk medium possesses a high degree of thermostability; e.g., when heated for 10 minutes to 90° at pH 7.0, about 72 per cent of its activity is retained. An enzyme obtained from a milk culture retained 48 per cent of its activity after heating to 110° but was inactivated at 115°. Proteases obtained on synthetic media showed no such thermostability, but were inactivated by 30 minutes' heating at 60°. The same observers note, in agreement with previous workers (E. D. Wilson and Haines), that the filterable proteases are not produced as a result of autolysis but are secretions of young intact cells. They showed, however, that polypeptidases and dipeptidases are, when present, intracellular enzymes and can be obtained in soluble form only as a result of autolysis.

ASPARTASE

The enzyme aspartase has been obtained in a cell-free condition from dried preparations of *B. fluorescens liquefaciens* by extraction with toluene water [Virtanen & Tarnanen (2)]. The kinetics of the reaction



have now been studied with the cell-free preparation. The extract was found to be mixed with fumarase



which could be removed by trypsin. The high specificity of the enzyme was confirmed, no other amino acids tried being deaminated, nor could the enzyme catalyse the uptake of ammonia by dicarboxylic acids closely related to fumaric acid, viz., mesaconic and *trans*-aconitic acids.

HYDROGEN LYASES

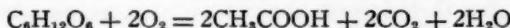
Hydrogen lyases, that is, enzymes liberating molecular hydrogen from formic acid or the sugars, have been shown to be adaptive, i.e. to be dependent on the presence of the substrate molecule in the medium on which the organism is grown (Stephenson & Stickland). By the use of resting suspensions of *Bact. coli* grown in formate broth, formic hydrogen lyase, catalysing the reaction



was studied. It was shown to be distinct from hydrogenase activating molecular hydrogen ($\text{H}_2 + \text{X} \rightleftharpoons \text{XH}_2$) and from formic dehydrogenase ($\text{H}\cdot\text{COOH} + \text{X} \rightleftharpoons \text{XH}_2 + \text{CO}_2$). The commonly held view that the hydrogen liberated from glucose is derived from intermediate formed formic acid has (for *Bact. coli*) been shown to be untenable; first because the hydrogen liberated from glucose appears as soon as the substrate and bacterial suspension come into contact and is subject to no lag as would be expected if formic acid had first to appear; secondly because the affinity of the enzyme preparation for formate is very much lower than for glucose. Moreover, it appears that two separate hydrogen lyases are concerned, one acting on formate and another on glucose, since it is possible to obtain a culture possessing the latter but in which the former is absent.

CARBOHYDRATE METABOLISM

A study on the carbohydrate metabolism of the *gonococcus* (Baron & Miller) reveals profound differences between this organism and members of the *Bacteriaceae*. Amongst the sugars tested (glucose, levulose, galactose, mannose, arabinose, and xylose), glucose alone was attacked. Oxidation studies in the Warburg manometer showed that glucose was oxidised to acetic acid. The oxygen uptake and carbon dioxide output agreed exactly with the equation:



The change was conclusively shown to be the work of three enzymes:

a) An *anaerobic glycolase* catalysing the change of glucose to lactic acid. This enzyme is relatively unstable, and slow in comparison with the other members of the system. It disappears on standing at room temperature or at 0°, in borate buffer at pH 9.0. It is remarkable that the degradation of glucose to lactic acid does not in this case go through methyl glyoxal, as is shown by the fact that methyl glyoxal is not oxidised by the organism.

b) *Lactic* (or rather *α-hydroxy-acid*) *dehydrogenase*, catalysing the reaction lactic acid → pyruvic acid. This is the most stable enzyme of the system.

c) *Pyruvic* (or *α-keto-acid*) *oxidase*, catalysing the reaction $\text{CH}_3\text{CO}\cdot\text{COOH} + \frac{1}{2}\text{O}_2 \rightarrow \text{CH}_3\text{COOH} + \text{CO}_2$. No evidence is forthcoming that pyruvic acid is broken down to acetic and formic acids as is the case with *Bact. coli*.

The enzyme systems of the *gonococcus* are much more restricted than those of, for example, the *Bacteriaceae*. Glucose alone of the common sugars is attacked, as are also the following common metabolites: formic, acetic, propionic, butyric, succinic, oxalic, and citric acids; glycine, leucine, alanine; acetaldehyde, ethyl alcohol, and glycerol. In addition to lactic acid, the following *α-hydroxy acids* are oxidised: *α-hydroxybutyric*, *glyceric*, *mandelic*, *glycolic*, and *malic*.

In growth experiments with haemolytic *Streptococci* on meat broth with 0.6 to 1 per cent glucose buffered with bicarbonate, about 72 per cent of the glucose was fermented to lactic acid, and no other products were found. No qualitative difference was noted between matt virulent and glossy avirulent cultures, but the growth was heavier and the rate of acid production consequently greater in the former case [Hewitt (1)].

Similar experiments with various strains of pneumococci yielded similar results, about 78 per cent of the glucose broken down appearing as lactic acid. Here it was found that glucose breakdown and growth were stimulated by phosphate, which was added in varying amounts to a medium already buffered with bicarbonate [Hewitt (2)].

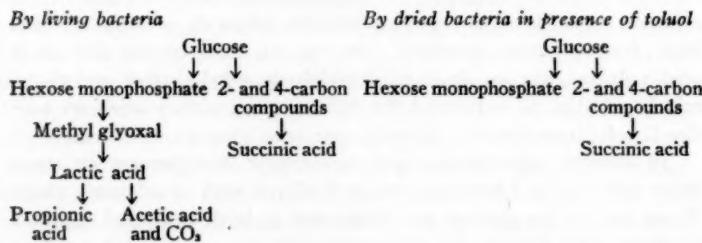
Termobacterium mobile has been further studied [Neuberg & Kobel (1, 2); Tanko] and is found to possess an outfit of enzymes consistent with its production of a typical alcohol fermentation.

Glyoxalase (ketoaldehyde mutase) and glycolase have been found in two lactic-acid bacteria, viz., *Str. casei* and *B. casei* [Simon (2)]. Glycolase has been found in *Cl. acetobutylicum* (Pett & Wynne).

Karström has found that by growing a strain of *Bact. coli* on a medium containing maltose, a preparation is obtained with a high fermenting power for maltose but none for saccharose. This appears to contradict the theory of Weidenhagen that saccharose and maltose are hydrolysed by the same enzyme, viz., α -glucosidase.

On the same lines as dihydroxyacetone, *l*-glyceraldehyde is fermented by *Bact. coli*, but much more slowly, giving a Cannizzaro reaction resulting in glycerol and glyceric acid, the latter passing to acetic and formic acids, the latter again to carbon dioxide and hydrogen. Glyceraldehyde differs from dihydroxyacetone in giving also about 25 per cent of inactive lactic acid; part of it must therefore be decomposed along the alternative path through methylglyoxal (Virtanen & v. Hausen).

The following scheme for the breakdown of glucose by propionic-acid bacteria is put forward by Virtanen & Karström.



These authors (in disagreement with van Niel) find that 10 to 15 per cent of succinic acid occurs in the fermentation of sugar but not of lactic acid. Fermentation by living bacteria gives, from glucose,

propionic and acetic acids and carbon dioxide in equimolecular proportions with succinic acid—from lactic acid the same without succinic acid. This suggests that succinic acid is not derived from glucose through a three-carbon compound since it is absent in the fermentation of lactic acid. Moreover, if it were derived from the condensation of two molecules of acetic acid, as is often suggested, this would result in the raising of the $\text{CO}_2/\text{acetic acid}$ ratio which, however, remains unity both in the glucose fermentation where succinic acid is produced and in lactic-acid fermentation where it is absent. Fermentations by dried bacteria in the presence of toluol bear out this view. Here the main products are hexose monophosphoric acid, on the one hand, and acetic and succinic acids with no carbon dioxide, on the other. Here the production of propionic acid is blocked by the inability of the preparation to ferment lactic acid. Supposing the succinic acid to be derived from acetic acid a corresponding one-carbon compound (carbon dioxide or formic acid) should appear, but none is found.

Pyruvic acid is broken down both by living and dried bacteria; by the latter only in the presence of phosphate, whence the absence of carboxylase may be inferred. The products from pyruvic acid are propionic and acetic acids, carbon dioxide, and succinic acid. The course suggested here is an oxidoreduction to propionic acid on the one hand and acetic acid and carbon dioxide on the other. The production of succinic acid from pyruvic acid alone amongst 3-carbon compounds is explained by assuming a preliminary condensation to a 6-carbon compound.

The production of acetyl methylcarbinol from sucrose by *Bact. subtilis* appears to take a course different from that postulated for yeast and the *Bacteriaceae*; that is, it does not come through pyruvic acid and acetaldehyde, nor is pyruvic acid decarboxylated to form acetaldehyde. The author suggests that it is derived directly from hexose by a split into a 2- and 4-carbon compound (Lafon).

STEREO-ISOMERISM AND ASSOCIATED GROWTH

A remarkable effect of associated growth on biochemical activity, first noted in 1926 (Pedersen, Peterson & Fred) is now amplified and confirmed (Tatum, Peterson & Fred). Four lactic-acid-producing bacteria, producing *l*-lactic acid, and three producing the *dextro* form were grown in association with *Cl. acetobutylicum* (13 strains). All seven lactic-acid bacteria then produced inactive lactic acid. The

peculiar property of influencing the rotation of the lactic acid produced by other organisms was not found to occur when other non-lactic-acid bacteria were used nor with strains of yeast tried; nor did it occur when the *Cl. acetobutylicum* was separated from the lactic producers by a viscose membrane, nor by cultures previously killed by heat.

THE ACETIC BACTERIA

Following up the observation that three strains of acetic bacteria will ferment sugar anaerobically in a manner closely resembling an alcoholic fermentation by yeast,² Neuberg & Simon now report a strain which is incapable of fermenting sugar anaerobically, in spite of the fact that the following enzymes are present:

glycolase (hexose diphosphate → methyl glyoxal) ;
glyoxalase and carboxylase.

Bact. Delbrücki has been found able to produce pyruvic acid in small yield from invert sugar and from calcium lactate [Simon (1)].

With regard to the mode of oxidation of isopropyl alcohol to acetone by *Bact. pasteurianum*, Müller (2) finds that, when acetone-treated organisms are used (*a*) with quinone, (*b*) with oxygen, as hydrogen acceptor, the rate of oxidation in the case of *b* is always from 25 to 30 per cent that of *a*. He deduces that either acetone treatment does not destroy the oxygen carrier (*Atmungsferment*) or the oxidation observed is direct and does not occur through an oxygen carrier. Moreover the action of *M*/10,000 cyanide reduces the oxidation only by one-half and *M*/100 by four-tenths.

The same author [Müller (1)] has shown that *Bact. pasteurianum*, either fresh or dead, oxidises ethyl alcohol and formaldehyde, the figures given being somewhat short of those required for the complete combustion claimed. Formic acid was oxidised only very slowly.

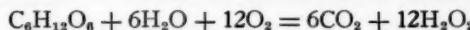
An extensive study of the influence of salts on the growth and formation of acetic acid by *Bact. acetigenoideum* on a medium containing ethyl alcohol and asparagine has been made by Krehan. Various combinations of the anions phosphate, chloride, and sulphate and the cations magnesium, calcium, potassium, and sodium were made. The results obtained are too complicated to admit of summarising, but are probably of great industrial importance.

² Cf. *Ann. Rev. Biochem.*, 1, 637 (1932).

The membrane of *Acetobacter xylinum* has been found to behave, in respect of X-ray diffraction, action of soda, nitration, and with Schweizer's reagent, like the cellulose of cotton (Khovine, Champtier & Sutra).

RESPIRATION

Bertho & Glück have worked on several strains of lactic-acid bacteria from which catalase is absent. Using glucose as hydrogen donator, they have shown that in the early stages the hydrogen peroxide formed is equivalent to the oxygen taken up, and during the same period the R.Q. is about 0.5 (0.45 for the first 30 minutes, 0.59 for the first 60 minutes). This is in accordance with the equation



When quinone or methylene blue are substituted as hydrogen acceptors the rate of oxidation is of the same order, viz., oxygen /quinone/M.B. = 1/4/2.5.

Toward inhibitors the three acceptors behave similarly: all are uninhibited by cyanide ($M/500$ to $M/5000$) and by carbon monoxide, but slightly inhibited by ethyl urethane and strongly by iodoacetic acid. The authors deduce that with this group of organisms oxidation by molecular oxygen occurs directly without the intervention of any *Atmungsferment* system.

An association of virulence with respiratory function has been shown by Todd to exist in the case of the haemolytic *streptococci*. Virulence is associated with the formation of matt (as opposed to glossy) colonies. Matt virulent cultures on repeated subcultivation on agar lose their virulence and finally produce smooth colonies. A high degree of virulence is associated with a high production of hydrogen peroxide and a low tolerance for the same. These characteristics lead to a dying off of the individuals possessing them and a favouring of the avirulent (smooth) types. Anaërobic cultivation also leads to loss of virulence. Hence to maintain virulence the culture must be kept aerated in the presence of added catalase. This treatment, however, will not raise virulence when once lost; this can be done by cultivating in broth and horse serum, kept aerated just below the point where hydrogen peroxide is demonstrable (Todd).

An investigation into the peculiar behaviour of *Azotobacter chro-öccum* toward oxygen (Meyerhof & Schulz) has revealed the fact that, whereas in the case of most tissues and bacteria very low ten-

sions of oxygen suffice to keep the *Atmungsferment* fully oxidised (Warburg & Kubowitz), in the case of this organism, lowering of the oxygen pressure below 15 per cent of oxygen results in part of the enzyme remaining in the reduced condition. This was shown in two ways. First, young cultures with high values of Q_{O_2} show a greater decrease of oxidation rate with falling oxygen pressure than do older cultures with lower values of Q_{O_2} .

TABLE III

Age of Culture (Days)	Q_{O_2} at 10°	Oxidation Rate in Percentage of That at 20 per Cent Oxygen Pressure		
		In 4 per Cent O_2	In 1 per Cent O_2	In 0.3 per Cent O_2
3	129	92	31	14
2	470	68.5	45	11
1	1,400	32	5	..
1	1,020	32	13	11

Secondly, the inhibition of the respiration rate by carbon monoxide is not in accordance with the equation for cases where the respiration enzyme is fully saturated with oxygen, viz.,

$$\frac{n}{1-n} \cdot \frac{PCO}{PO_2} = K,$$

where n is the residual respiration and $1-n$ the inhibition due to carbon monoxide. In the case of *Azotobacter*, at low concentrations of oxygen the inhibition by carbon monoxide is less than that demanded by the equation above and falls off with increased values of Q_{O_2} and decreasing oxygen tension. In other words the fact that the respiratory enzyme is unsaturated with respect to oxygen results in part of the available carbon monoxide being expended in saturating the enzyme rather than in replacing oxygen from the enzyme surface.

During the passage of gasworks liquors through sewage beds, organisms containing an indophenol oxidase system increased relatively to the others. Of such organisms 80 per cent consisted of a gram-negative *vibrio* able to grow aerobically on a synthetic medium with 0.04 per cent of phenol as sole source of carbon. *Vibrio tyrosinatica* was sought for, but not found (Happold & Key).

Various porphyrin compounds have been obtained from *B. diphtheriae* and from *B. phosphorescens*. The details of these belong to another section (Coulter & Stone; Stone & Coulter).

PUTREFACTION

The action of mixed putrefactive bacteria on arcain (1,4-diguanido-*n*-butane) is to produce carbamyl agmatine, agmatine, carbamyl putrescine, putrescine, and urea (Linneweh).

SPORE FORMATION

Working with *Bact. mycoides* in a peptone medium Brunstetter & Magooon found that the percentage of spores decreased both with concentration of medium and with aération. Tarr, working with *Bact. mesentericus*, *subtilis* (2 strains), and *megatherium*, has shown that the use of either casein digest containing 208 milligrams of amino N. per 100 cubic centimeters, or beef digest at 101 milligrams per 100 cubic centimeters, causes sporulation to be either absent or very low. By diluting serially, 98 to 99 per cent of spores can be obtained at the lowest concentrations used (1 in 30 for the tryptic casein and 1 in 10 for the beef medium). Tarr attributed the absence of sporulation to a high concentration of amino nitrogen. He showed that complete sporulation could be obtained on a synthetic medium where carbon and nitrogen were supplied by sucrose and ammonia respectively. The same synthetic medium with carbon and nitrogen supplied by known mixtures of amino acids gave complete sporulation at the lowest concentrations and 2 to 0 per cent at the highest.

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IMMUNOCHEMISTRY*

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As in last year's review, papers which do not deal in some way with the chemical basis of immunology have been excluded. Space requirements have made it necessary to eliminate many others of undoubtedly interest. The period covered is essentially that from December, 1931, to November, 1932, and the subject is treated under two main headings: I. The Chemistry of Immune Substances; II. The Chemistry of Immune Reactions.

I. THE CHEMISTRY OF IMMUNE SUBSTANCES

1. NATURALLY OCCURRING PROTEINS AND PROTEIN DERIVATIVES AS ANTIGENS

Fowl livetin has been separated from egg yolks by Jukes & Kay (1). In anaphylaxis and precipitin tests it showed a close relationship or identity with fowl-serum globulins. According to Proca (2), beef-choroid melanin showed antigenic properties only in albino rabbits, yielding sera giving complement fixation with beef, pig, sheep, and chicken melanin. Rabbit melanin also produced similar antibodies in albino rabbits. It is interesting in this connection that Waelsch (3) found that the pigment fraction of melanin is firmly linked to a protein of distinctive properties.

Kirk & Sumner (4) have shown that jack bean urease antiserum inactivates soy bean urease and protects rabbits against soy urease, so that the enzyme appears to be identical in the two species of bean.

Among the bacterial proteins, an interesting product has been extracted with acid alcohol from S- and R-forms of the *Salmonella* group by P. B. White (5). The protein is insoluble in neutral or alkaline alcohol and soluble in dilute alkalies or hydrochloric acid. Its solutions are precipitated by salts and give typical protein reactions. It is antigenic, yielding precipitating sera which agglutinate S-forms least readily. The same product appears to be present in the coliform,

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dysentery, and proteus groups. Its removal interferes with somatic agglutination, possibly owing at least in part to its ready salt-precipitability. In the same group, Olitzki (6) has found it possible by cataphoresis of *Proteus X 19* extracts at pH 4 to obtain the "O" antigen at the positive compartment, leaving the "H" antigen in the middle vessel.

As regards tubercle bacillus proteins, Seibert & Munday (7) have carried still further the purification of unheated tuberculin, finding that the active protein may be freed from almost all accompanying polysaccharide by precipitating and washing with trichloroacetic acid. Seibert (8) has also shown that if sufficiently large amounts of the purified tuberculin protein are used, animals can be sensitized to it in the same way as by infection with tubercle bacilli. Heidelberger & Menzel (9) isolated a series of fractions from the timothy-grass bacillus analogous to those previously obtained from *Streptococcus hemolyticus* by the same method.

Considerable interest has been shown in the heterogenetic antigens of bacteria. By absorption experiments Eisler (10) has found these to be different from the Forssman antigen in guinea-pig kidney and also to differ from one bacterial species to another. [See also Eisler & Howard (11) and Landsteiner & Levine (12).] On the other hand, Bailey & Shorb (13) found that the anti-sheep hemolysin produced by injecting Type I pneumococci into rabbits appeared to be the same as that induced by boiled sheep corpuscles. Moreover, rabbits immunized with sheep red cells were relatively resistant to infection with the pneumococcus. Further work is needed to clear up inconsistencies.

Ramon (14), in a discussion of the nature of diphtheria toxin, has concluded that all its properties can be explained on the basis of a single substance, rather than the complex mixture postulated by Ehrlich. Studies on the purification of this toxin by adsorption are grouped under (15); papers on the influence of the medium on the potency of the toxin under (15a), and those on the destructive or preservative action of various substances under (16). Moerch (17) believes that diphtheria toxin contains an unstable "flocculinogen," which influences the rapidity of flocculation of toxin-antitoxin mixtures and may occur in the filtrate from non-toxigenic strains. However, the experiments do not seem to have been controlled by dilutions with broth alone, which, according to Bunney, favorably influences the K_t . Hirsch (18) reports that the toxin extractable from dried,

defatted diphtheria cells appears identical with that in the broth and is given off to water at once, while the other nitrogenous extractives dissolve more slowly. The amount of toxin liberated increases with the age of the culture, as does also the amount of inorganic phosphorus, pointing to the enzymic degradation of a precursor.

For the influence of various substances on tetanus toxin see (19), on botulinus toxin (20), and on vibrio toxin (21).

Acetic-acid-precipitated staphylococcus toxin was shown by Burnet & Freeman (22) to be quite stable, especially at pH 7. Their best preparations contained 36,000 units per mg. of nitrogen. Formaldehyde detoxifies the toxin at a rate proportional to the concentration, the square root of the hydroxyl-ion concentration, and with a high temperature coefficient. In slow reactions in alkaline solutions an intermediate, reversible toxin-formaldehyde product is formed. Apparently only a single toxin is involved.

The action of formaldehyde on meningococcus toxin has been found by Klein (23) to be much like that on diphtheria toxin. Malcolm & B. White (24) have studied the endotoxin of meningococcus. It is antigenic and independent of the acetic acid-precipitable or heat-coagulable proteins of the micro-organism.

Presumably the type-specific antigen postulated by Enders as distinct from the specific polysaccharide (25) is being studied by Felton (26) in the form of an apparently protein- and carbohydrate-free product which contained nitrogen and protected mice type-specifically in doses as low as 0.004 mg. The homologous specific polysaccharide had an inhibiting effect.

The lysinogenic properties of bacteriophage have been found independent of biologically demonstrable bacterial protein by Bossa (27), and have also been studied by Kligler & Olitzki (28). Legroux & Djemil (29) pointed out similarities of phage to enzymes, and found it to be inactivated by formalin but still capable of giving rise to anti-phage in rabbits. Krueger (30) has published a critique of the serial-dilution method. An expression similar to one derived by Krueger for the kinetics of the binding of phage by bacteria has been found by Schlesinger (31) in an extensive investigation which includes the calculation of the particle size by two independent methods giving concordant results considerably at variance with those of ultrafiltration experiments.

Murphy and his collaborators (32) have found that the highly purified causative agent of a chicken tumor (33) gave rise only to

neutralizing antibodies. Analogies are pointed out between the tumor-producing agent and the "mutagen" of *Pneumococcus*, that is, the substance responsible for the change of an R-form into an S-form of the same or a different type (34).

2. CHEMICALLY ALTERED PROTEINS AS ANTIGENS

A study of the effect of alkali on the antigenic properties of proteins by Johnson & Wormall (35) disclosed that the precipitability of horse serum by antibody was markedly diminished at 19° and pH 12. If not too much alkali was used the treated serum could still be converted into an antigen by nitration, or by iodination—further evidence that the tyrosine groups in proteins play an important part in determining their antigenicity. Jacobs' (36) studies on iodinated sera as antigens showed that much weaker iodine solutions than Lugol's can cause the formation of iodo-antigen, pointing to the possible allergic origin of certain iodine disturbances in man.

Medveczky & Uhrovits (37) found that in benzoylating various sera and gelatin the products were soluble chemospecific antigens if the process was not pushed to the limit. Cross-reactions with other acyl proteins were observed; benzoyl gelatin (best containing 5 per cent of benzoyl) reacted to higher dilutions with the antisera than did the other proteins; benzoylated typhoid organisms gave the best antisera, and the precipitin reactions were not inhibited by sodium benzoate. Since the benzoylated antigens still reacted slightly with sera prepared from the native proteins, small amounts of unaltered protein might have been present. Formalin was found by Puccinnelli (38) to deprive eel and beef sera of their hemolytic properties with only a 50 per cent reduction in toxicity and no diminution in precipitinogenic power.

The distribution of intravenously injected antigen in rabbits was studied by Haurowitz & Breinl (39), using atoxylazo-horse serum. Most of the arsenic disappeared from the blood in six hours, at which time 25 to 30 per cent was recoverable from the liver and an equal amount from the marrow-containing bones. One-third of the arsenic was eliminated in the urine in 24 hours.

3. HAPTENS

a) *General.*—The relation between structure and specificity has been considered by Erlenmeyer & Berger (40) on the basis of

Grimm's theory of "*Feldwirkung*," or molecular fields (42). Because of the close serological relation existing between *p*-PhOC₆H₄NH₂, PhNHC₆H₄NH₂, and PhCH₂C₆H₄NH₂, when coupled by the diazo reaction with horse serum, it is concluded that haptens differing only in groupings whose fields of force are equal are indistinguishable by immunological means. The evidence presented loses in force because the technique used did not eliminate the possibility of cross-reactions due to unchanged horse serum, and since cross-reactions were obtained between PhCOC₆H₄NH₂ and the -NH and -O- haptens, although the force fields of the -CO- compound are quite different. In later work (41) the antimonic acid radical is shown to differ from the immunologically and chemically similar -PO₃H₂ and -AsO₃H₂ groups, and union of hapten and antibody *in vivo* is shown to occur.

Continuing their studies on conjugated carbohydrate proteins, Goebel, Babers & Avery (43) have synthesized the α - and β -*p*-aminophenol glucosides, coupled them with protein, and studied their immunological reactions [Avery, Goebel & Babers (44)]. While their previous work showed that β -glucosides of *different* sugars yielded rigidly specific antigens, it was now found that the α - and β -glucosides of the *same* sugar possessed antigenic properties in common, showing cross-precipitation in the antisera. However, in an α -glucoside horse-globulin antiserum, an excess of α -glucoside inhibited the specific reaction between antibody and α -glucoside chicken serum, while the β -glucoside did not. Similarly, in the β -antiserum the β -glucoside inhibited the homologous reaction, while the α -compound did not. These findings strikingly parallel the polysaccharide cross-reactions between Type II pneumococcus and the Type B Friedlander bacillus.

Landsteiner & van der Scheer (45) have again shown serological differences between steric isomers. These workers (46) have also prepared *p*-aminobenzoyl-glycylglycine and the similarly acylated glycyl-leucine, leucyl-glycine, and leucyl-leucine, and have coupled them with proteins. Each possessed a characteristic specificity, although there was some crossing between the glycyl-leucine and leucyl-leucine derivatives, indicating that the specificity depended more on the structure of the terminal amino acid carrying the free COOH group than on the other component of the dipeptide. Analogies with enzyme action are pointed out. The same workers (47) have prepared haptens by coupling *p*-amino-succinanilic, -adipanilic, or suberanilic acids with tyrosine or resorcinol. These actually precipi-

tated antisera to the homologous azo protein. Suberanilic-azo-resorcinol, which contains two azo groups and a long aliphatic chain, reacted at as high a dilution as 1:1,000,000.

b) *Specific polysaccharides.*—A study of physico-chemical properties in this group by Heidelberger & Kendall (48) was a preliminary to attempts to determine molecular weights by the viscosity and diffusion methods (49). The salts of the specific polysaccharide of Type III pneumococcus (S III) are highly ionized and exhibit anomalies in viscosity and diffusion of very great magnitude, owing to the strongly acid nature and peculiar structure of the multivalent anion. These anomalous viscosity effects are smaller in other, more weakly acidic, specific carbohydrates. Corrections applied to the data of Babers & Goebel (50) on the molecular weight of S III changed their value from 118,000 to 2,400. A method was developed, applicable to any specific polysaccharide, for the quantitative determination of S III, based on the amount of nitrogen specifically precipitated from an antibody solution calibrated with known amounts of S III in the region of excess antibody (51). With the aid of this method a tentative formula weight of 5,600 was assigned to S III, and it was concluded that the formula weights of the specific polysaccharides as a group do not exceed 10,000.

Goodner, Dubos & Avery (52) have used the S III-splitting enzyme in rabbits, and found that the animals may be cured of an otherwise fatal intradermal infection with Type III pneumococcus if sufficient enzyme is given intravenously. Dubos (53) has studied the factors affecting the yield of enzyme, finding that it is produced by the soil bacillus only when S III is present in the medium, that yeast extract is the best adjuvant, and that an accompanying toxic product may be adsorbed by means of Type "C" aluminium hydroxide at pH 5.5. Ward (54) has given evidence of a possible unstable, highly antibacterial intermediate product between the S III antigen of the intact pneumococcus cell and the S III as isolated.

Specific carbohydrates of the typhoid-paratyphoid group have been found by Meisel & Mikulaszek (55) to be distributed in much the same way as the heat-stable agglutinogen. The S- and O-polysaccharides appeared identical, the R- and S-products different. Tomcsik & Szongott (56) failed to find a relation between specific polysaccharide content and virulence or presence of a capsule in anthrax bacilli. However, the encapsulated organisms gave more of a nitrogen-containing fraction, much of which was precipitable by

copper sulfate and was therefore unwarrantedly considered to be protein. Unfortunately, in both of these studies the specific polysaccharide agar was used as a culture medium and therefore presumably contaminated the products isolated.

In the tubercle bacillus group, McAlpine & Masucci (57) found the polysaccharide in unheated tuberculin from a human strain to be very sensitive to acid, even at ordinary temperatures. The *d*-arabinose component is considered the portion conferring reactivity with serum. Masucci, McAlpine & Glenn (58) found the polysaccharide in tuberculin from a bovine strain to contain no pentose and to show little cross-reaction with antibodies to the human type polysaccharide, differing in both respects from the main cellular carbohydrate fraction of the same bovine strain.¹ Heidelberger & Menzel (59) have isolated two independently specific polysaccharides from the complex carbohydrate mixture in the human type tubercle-bacillus cell, one of which, at any rate, seems also to occur in the bovine, avian, and timothy-grass types. The fractions are characterized by differences in optical rotation and in their ease of hydrolysis. Both carbohydrates occur in the human type tuberculin.² Gough (60) has attempted to identify the acid products of hydrolysis of the specific carbohydrate material and concluded that an acid similar to glycollic acid was present. H. du Mont & Anderson (61) removed all of the phosphorus from their specific polysaccharide material by purification over the acetyl derivative.

Morgan (62) has further described the specific carbohydrate of *B. dysenteriae* Shiga as a weak acid with $[\alpha]_{Hg\ green} = +107^\circ$, a specific reactivity as high as 1:12,000,000, and a nitrogen content of 1.8 per cent. Galactose and an acetylaminohexose are obtained on hydrolysis. The specific polysaccharide could be determined with a fair degree of accuracy by comparison of an unknown with the minimum amount necessary to kill passively sensitized guinea pigs.

Sordelli & Mayer (63) have confirmed their earlier work on cross-precipitin reactions with agar and have shown that the precipitins to it in anti-anthrax serum prepared from agar-grown organisms are independent of the true anthrax precipitins. Zozaya (64) has found that dextran, a simple polyglucose produced by *Leuconostoc mesenteroides*, gives precipitates in many bacterial antisera, but

¹ Unpublished results by the reviewer and A. E. O. Menzel.

² *Ibid.*

that homologous antibodies are not absorbed. With Wood (65) he has recorded other cross-reactions, as has Savino (66) with a specific polysaccharide from *Staphylococcus aureus* grown in peptone-glucose broth.

The possible antigenic function of specific polysaccharides has again been studied. Zozaya (67) has reported that approximately 0.03 mg. of anthrax polysaccharide (containing agar?) adsorbed on collodion particles can produce precipitins in rabbits. Similar results were obtained with specific polysaccharides of other organisms, but no data are given on the purity of the preparations used. Pneumococcus polysaccharides did not react,³ except in a horse. Casein and aluminium hydroxide particles were also effective carriers, but the polysaccharides alone, in 1:10,000 solution, failed to induce antibody formation. Zozaya explains the failure of collodion particles coated with immune serum to agglutinate in homologous polysaccharide solutions on colloid-chemical grounds. The reviewer believes agglutination fails owing to the soluble compound formed by the relative excess of polysaccharide much as in the agglutination inhibition discussed by Francis (see below).

That the heterogenetic hapten or haptens belong to the polysaccharide group rather than to the lipoids now seems increasingly evident (10, 11, 12).

c) *Lipoids*.—Additional experiments on highly purified lipoids have at last been carried out [cf. also (68)]. Weil & Besser (69) have shown that antibodies to cholesterol (*a*)—pig serum fail to react with dihydrocholesterol (*b*), or cholesterol dibromide, oxide, acetate, or palmitate. Antibodies could not be produced with the last four, but (*b*) yielded antisera of high specificity. The conclusion was reached (70) that chemically defined lipoids are comparatively non-reactive as antigens. An investigation by Berger & Scholer (71) included: (*a*) ; (*b*) ; ergosterol (*c*) ; irradiated ergosterol (*d*) ; crystalline vitamin D₂ (*e*) ; phytosterol ; sitosterol (*f*) ; and analogous substances. Alcoholic solutions were mixed with pig serum for the injections, and great care was taken in controlling as many difficulties as possible. A very low percentage of rabbits responded with antibody formation ; those injected with (*e*) died of intoxication ; 8 of 17 on (*a*) died of anaphylactic shock, although no (*c*) rabbit died. The

³ S II and S III were not adsorbed in detectable amounts by collodion particles in experiments made by the reviewer.

lipoid-antibody content roughly paralleled the pig-serum-antibody titer. Flocculation reactions could not be obtained. An antiserum to (c) fixed complement only with c, d [which still contained (c)], and (f); but (c) reacted only with homologous antiserum. Antisera to (a) reacted only with (a), (b) (contrary to Weil & Besser), and in one case, with (d). It is concluded that pure lipoids may, through the "combination" method, stimulate the formation of antibodies with a high degree of specificity. The broad range of reactivity of alcoholic organ extracts would thus be due to the complexity of the mixtures used.

Work on the chemical nature of the antigens (haptons) of alcoholic brain extracts has been done by Rudy (72), who has also separated mixtures of lipoid haptons by differential adsorption (73). Plaut & Rudy (74) have made lengthy studies of the "masking" by serum lipoids of the power of alcoholic brain extracts to fix complement with antisera. The reviewer believes that the effect may be simply explained as an inhibition of the not very specific reaction by the excess of non-specific lipoids normally present in serum. The "masking" effect of lecithin on complement fixation by cholesterol-anti-cholesterol was also studied. The broad specificity of the serum lipoids of numerous animals was noted by Ishikawa (75). Gonzales, Armangué & Morato (76) found that while lipoids *per se* did not stimulate antibody production, adsorption on animal charcoal rendered them antigenic. These workers consider lipoids to be true antigens when present in the proper physical state. The antigenic properties of the floccules in human Wassermann-positive sera are discussed by Eagle (77).

A preliminary study of isohemagglutinogens has led Schröder (78) to consider them as lecithin analogs. These may easily be impurities, however, especially since Landsteiner (79), as well as Freudenberg and Brahn and their co-workers (79) believe specific carbohydrates to be involved.

4. ANTIBODIES

The normal agglutinins for many bacteria in blood and urine may be concentrated 30,000-fold, according to Freund & Katz (80), by adsorption and elution, and mice may be protected against infection by small amounts of the concentrate.

In addition to speculations on the mode of formation of anti-

bodies (81) more evidence has appeared against the actual occurrence of antigen fragments in antibody. Berger & Erlenmeyer (82) failed to find arsenic in as much as 30 cc. of antiserum to atoxylazo-horse serum. An excellent review and discussion are given. Similar results were obtained by Hooker & Boyd (83) with atoxylazo-casein estimated to contain about 100 As groups in the molecule. Sox & Manwaring (84) interpret a series of experiments as a test-tube synthesis of new, intermediary, or hybrid specificities. Velluz (85) reports himself unable to confirm Salkowski on protein-free antitoxin, and A. Schmidt & Tuljtschinskaja (86) have found typhoid agglutinins to be almost completely destroyed even by a 1 to 25 dilution of active gastric juice in 2 hours at 37°.

Reiner & Reiner (87) have found in normal horse serum, fractions which are not specifically reactive, but are similar to those encountered in the purification of anti-pneumococcus serum by Felton. This worker (88) has been able to dissociate a portion of the specific precipitate of Pneumococcus I and II polysaccharides and antibody by solution in lime-water and addition of phosphate and calcium chloride to adsorb the polysaccharide. Felton has also further purified the water-insoluble pneumococcus antibody protein by removal of an inactive fraction with AlCl_3 at pH 5.2, or with ZnCl_2 at pH 7. The metal salt of the protein in the supernatant liquid was completely precipitable by specific polysaccharide, but only 80 to 90 per cent precipitated when freed from metal with carbonate or phosphate. The same was true of antibody protein recovered by dissociation. Pure antibody in amounts sufficient for chemical characterization would therefore seem almost at hand.

References to the preservation and stability of antibodies are grouped under (89). References to the distribution of antiviral and other antibodies among the serum fractions are given under (90). The stimulating action of tapioca on the production of precipitins has been shown by Liggeri (91), and of tapioca and calcium chloride on antitoxin production by Ramon & Lemétayer (92), although there is no agreement as to the mechanism involved. Hektoen & Delves (93) have investigated the relative independence of precipitins in polyvalent antisera.

In standardizing Type I anti-pneumococcus serum, W. Smith (94) has found a modification of the Dean-Webb optimal-proportions flocculation test to give a high degree of correlation with mouse protection, but to yield low values in the case of antibody solutions. The

quickest flocculation occurred at the equivalence point.⁴ Duplicate quantitative analyses of specifically precipitable nitrogen, as proposed by Heidelberger, Sia, & Kendall (95), are avoided but approximately one hour and twenty minutes of manipulation are required for each serum,⁵ while the analytical method requires only 40 minutes of manipulation in the case of a single serum and 25 minutes for each additional serum analyzed at the same time. Zozaya (96) has applied his method of standardization, also based on precipitation with specific polysaccharide, to anti-dysentery Shiga serum and to anti-meningococcus serum. A dilution method is used by Glenny & Barr (97) in comparing the avidities of antitoxins. [See also Moerch (17 and 98) on this point.] Electrodialysis is said by Gerlough & W. White (99) greatly to improve the stability of diphtheria- and tetanus-antitoxin concentrates, removing a small fraction rich in lipoid and phosphorus.

II. THE CHEMISTRY OF IMMUNE REACTIONS

a) *General.*—Sachs & Behrens (100) report a series of complex "model" experiments, many employing tannin, which are interpreted as failing to confirm the theory of Stern and work of Reiner and of Freund, that the action of antibody on antigen is one of dehydration.

Marrack & Smith (101) have confirmed the chemical combination of hapten with antibody by dialyzing uncombined hapten—in this case atoxylazotyrosine—from mixtures with normal globulin and with antibody to atoxylazo-horse globulin, finding in the latter case up to a twenty-fold increase in the ratio of bound to free hapten. Adsorption was ruled out since the antibody failed to bind more of the hapten-like methyl red than did normal globulin. Failure to consider the fundamental chemical union between hapten and antibody has led Ward (102) into error in discussing the effect of Type I pneumococcus antiserum in pneumonia. A dose of 200 cc. of serum would neutralize the amount of S I in only about 500 cc. of culture, not 12,500 liters, as calculated by Ward.

b) *The precipitin reaction.*—The use of this reaction in the quantitative estimation of a specific polysaccharide (51) and in the stand-

⁴ This term is suggested by the reviewer as preferable to "equilibrium point" or "neutralization point."

⁵ Reviewer's calculation.

ardization of antisera (94, 96) has been mentioned. Culbertson & Seegal (103) have employed it for the determination of antibody in anti-egg albumin serum. Applying Heidelberger, Sia & Kendall's method (95) it was found that the ratio of egg albumin to antibody precipitated at the "neutralization point" (at which neither antigen nor antibody could be demonstrated in the supernatant liquid) was 1:13. A series of small tests is set up with increasing amounts of a solution of crystalline egg albumin, of which mg.-at-the—"neutralization-point" $\times 13 =$ antibody protein. A similar method, based on the Dean-Webb optimal-proportions procedure, has been advanced by Taylor, Adair & Adair (104) for the estimation of proteins.* Crystalline egg albumin and horse globulin were used. Culbertson (105) has been able to determine the blood volume of rabbits by the precipitation method. Masuda (106) has studied the speed of formation of the specific precipitate, as has also Eagle (107) in the course of a study of the factors influencing the rate of immune aggregation reactions in general. The results are considered to support Eagle's theory of the mechanism of aggregation reactions, but since the effects are in the direction demanded by any simple chemical reaction, their bearing on the theory is not clear. The influence of salts on the precipitin reaction has been studied by Downs & Gottlieb (108).

c) *Agglutination*.—Francis (109), studying the effect of specific polysaccharide on agglutination of pneumococci, has found an extensive parallel with the precipitin reaction and concludes that both reactions are expressions of the same chemical mechanism. Bier (110) has found the "agglutination optimum" to shift to lower serum dilutions with increasing concentration of salt.

d) *Anaphylaxis*.—Neill, Sugg & Richardson (111) have passively sensitized guinea pigs with diphtheria antitoxin and shocked them with toxin, considering the effect as a typical anaphylactic shock produced by the toxin itself and not by nucleoprotein or polysaccharide. Meyers (112) has shown that fibrinogen is absent in many antibody concentrates capable of causing serum disease, so that it cannot be the causative factor. Gebauer-Fuelnegg, Dragstedt & Mullenix (113) have found in the lymph and blood of dogs during anaphylactic shock a dialyzable substance giving the reactions of histamine.

* The method given in (51) is also applicable to proteins.

e) *Hemolysis*.—H. von Euler & Gard (114) found the normal hemolytic power of sheep serum toward rabbit cells to be due to a typical "amboceptor" (*A*) and a second component, apparently the mid-piece of sheep complement. H. von Euler & Brunius (115) have studied the effect of pH and salt concentration on the union of red-cell stromata and lipoids with (*A*), as well as of the elution of (*A*) from the complex at alkaline reactions. The speed of the reaction was studied, also the amount of hemolysis in constant time at different (*A*) concentrations. Ponder (116) has continued his work on the kinetics of hemolysis, introducing correction terms for the velocity of mixing and for the inhibition of hemolysis by the hemoglobin liberated. The experimental procedure and reasoning are difficult and involved, although orderly and apparently reasonable if the assumptions made are granted. It was found, under the conditions used, that in the lysis of a given number of cells a constant amount of complement is used up irrespective of the amount of (*A*) used for sensitization. Olitzki (117) observed that silica gel removes anticomplementary material from serum and may be used for the adsorption of antibodies.

f) *The toxin-antitoxin reaction*.—H. Schmidt, Scholz & Perry (118) have made quantitative studies on the dissociation of the diphtheria and tetanus toxin-antitoxin complexes by formalinized toxin. The evidence is considered in favor of the Bordet adsorption theory, but the arbitrary conditions chosen raise doubt as to whether equilibrium was established. On this work H. Schmidt & Scholz (119) have based a method for the evaluation of formol toxoids, as well as the opinion that toxin possesses a greater affinity for antitoxin than does toxoid, a conclusion exactly the opposite to that reached by S. Schmidt and Ramon from a study of the same reaction. The situation thus supports the reasoning of Heidelberger & Kendall that the findings are characteristic of a true chemical equilibrium in which affinity relations affect only the extent of dissociation.⁷ Hansen (120) has modified Ramon, Legroux & Schoen's method of dissociating the diphtheria anatoxin-antitoxin precipitate by heat. Fifteen minutes in boiling water containing 2.4 per cent of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ sufficed to yield 50 to 90 per cent of the anatoxin of a 200-300-fold degree of purity. This could be increased to 400-fold by adsorption on alumina followed by elution. The prod-

⁷ Cf. *Ann. Rev. Biochem.*, 1, 669.

uct, however, still contained enough horse-serum protein to shock sensitized guinea pigs.

The reaction between staphylococcus toxin and antitoxin has been analyzed further by Burnet (121). At the flocculation optimum an excess of antitoxin is present. The quantitative data presented are considered best explained by adsorption of excess antitoxin by the precipitate, although in the case of the anatoxin it is considered that combination may occur in the proportions TA and T₂A.

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THE BIOCHEMISTRY OF THE FUNGI*

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In studying recent literature one cannot but be surprised at the number of works on the physiology and biochemistry of mould fungi. It must be said that in availing ourselves of these organisms it has been possible to solve a series of problems reaching far beyond the fungi themselves.

At the present time, more than ever, one ought to speak less of the biochemistry of the separate fungi species, but more of their races or strains, as the study of the latter has been invaluable in throwing light on the metabolic processes of fungi. It suffices to recall the works of Butkewitsch, Chrzaszcz, and Bernhauer, on the formation of organic acids in fungi, in order to understand how important it proved to be to turn to the separate species and races of the mould fungi for an understanding of the formation of most diverse organic acids from sugar. It is this biochemical variation of the fungi races which is responsible for the contradictory data of the various authors working with these fungi. For instance, in a given fungus species investigated by different authors, sometimes nitrogen fixation is observed, while sometimes it fails to take place. This contradiction may be explained, perhaps, by the existence of racial differences in metabolism. But even in availing one's self of the same fungus race the conditions of cultivation may lead to the loss of certain biochemical characters and the appearance of others. The theory of variation, well elaborated in regard to bacteria, should be applied also to the biochemical study of the fungi.

ON THE NUTRITION OF FUNGI

Mould fungi serve well for studying the suitability of different carbon compounds for the building up of living cells. It was proved long ago that fungi are able to synthesize their cell substance from carbon compounds of the most diverse sorts. Earlier authors approached the problem from one point of view only; they judged of the value of the carbon substance only by the yield of mycelium or

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the rate of growth of the fungus. In the recently published work of Tamiya (from the laboratory of K. Shibata) this question has been subjected to fresh intensive study, on the fungus *Aspergillus oryzae*. One hundred and twenty-three chemically pure organic preparations were tested. Not only the growth of the fungus from spores was studied, but also the growth and respiration of the mycelium, as well as the anaërobic respiration of the fungus, which the author identifies with ordinary alcoholic fermentation. Out of the 123 substances tested, 51 proved suitable for the respiration and growth of the mycelium, 8 only for respiration, but not for growth, while 64 compounds exerted no influence. But growth proved possible only when the fungus had utilized the source of carbon given to it, as material for respiration. According to the data of Tamiya, the best sources of carbon for *Aspergillus oryzae* are carbohydrates and polyatomic alcohols (glycerol, mannitol, inositol, etc.). Alcohols of the aromatic series, as well as monoatomic alcohols of the aliphatic series, are of no use for fungi, with the exception of ethyl alcohol, which is a good material for respiration and growth. Among the acids, those capable of assimilation are: succinic, lactic, malic, citric, salicylic, and several others. Aldehydes, ketones, and esters proved unsuitable. The author has established that the groups CO— and CHOH— are better assimilated than either the CH— or the CH₂— group, which, by the way, has been pointed out before for other fungi. Pyruvic acid was utilized better than lactic acid, which, in turn, surpassed propionic acid. The introduction of a methyl or methylene group as substituent lessened the utilizability of the compound. Thus itaconic acid [HOOC·CH₂·C(:CH₂)·COOH] was assimilated less satisfactorily than succinic acid, and mesaconic acid [HOOC·CH: C(CH₃)·COOH] less than fumaric acid.

Tamiya has shown that the compounds assimilated by the fungus possess certain characteristic atomic groups (*Hauptradicale*) at the end of the chain or in the ring: CH₃·CHOH—, =CH·COH=, CH₃·CO—, CH₂OH·CH₂—, etc. If the combination does not contain one of the *Hauptradicale* it is not assimilated by fungi. On this basis Tamiya comes to the important conclusion that the combustion of the carbon source in its first phase is connected with the splitting by oxidation-reduction of the *Hauptradicale*.

In anaërobic respiration, identified by the author with alcoholic yeast fermentation, Tamiya has shown that a series of alcohols and organic acids, well utilized by the fungus in aërobic metabolism, are

at the same time not fermented at all. This observation is at variance with the views held by Kostytschew (1908-1921) to the effect that organic compounds such as quinic acid, tartaric acid, lactic acid, and mannitol may be split by *Aspergillus niger* into alcohol and carbon dioxide, after a preliminary conversion into glucose. The views of Kostytschew have also been refuted by Butkewitsch.

Important data on the decomposition of chemically stable compounds have also been published. Tausson (1, 2) reports that micro-organisms may use higher paraffins as sources of carbon, which agrees with the older observations of Miyoshi (1895) and Rahn (1906) on the utilization of paraffin by *Penicillium*. A series of other authors [Zikes (1925)] have shown that the breakdown of the paraffins takes place even after they have been first freed from admixture with other organic substances which might induce growth. However, these contaminants can scarcely be of great importance, since the actual consumption of paraffin is rather considerable, as may be seen from the following table of Tausson:

Organism	Duration of Culture days	Consumption of Paraffin mg.	Mycelium Formed mg.	Economic Coefficient
<i>Aspergillus flavus</i> F.....	35	591.4	289.1	48.8
Fungus A (not fully identified) ..	35	348.6	277.8	79.7
Fungus D (not fully identified) ..	35	292.5	112.0	38.3
<i>Actinomyces</i> A.....	30	284.7	209.7	73.6
<i>Actinomyces</i> B.....	30	477.5	301.4	63.1

It should be noted that with paraffin the economic coefficient¹ is particularly high, which is to be expected since its heat of combustion is considerably greater than that of other organic compounds. The rate of consumption of the paraffin depends on different conditions, such as the nitrogen source, pH, the age of the fungus, etc.

According to the data of Tausson, *Aspergillus flavus* consumes not only paraffin, but also bees' wax, cocoa-butter, tripalmitin, and other such compounds, with an increase in substrate utilization and a decrease in the economic coefficient. Tausson has established that the two species of *Actinomyces*, A and B, decompose cholesterol, besides paraffin, with an economic coefficient of 63 to 68.

¹ I.e., the ratio between the dry weight of the mycelium formed and the amount of paraffin consumed.

Hopkins & Chibnall have shown that the fungus described by them under the name *Aspergillus versicolor* may grow on higher paraffins of definite structure, with chains not exceeding $C_{34}H_{70}$, as the only source of carbon. The authors have tried to determine the intermediate products of breakdown in cultures on $C_{27}H_{54}$, but only the end-product, CO_2 , was obtained. There are indications that the primary products of the oxidation of paraffins are ketones, which on further oxidation give rise to fatty acids with a shorter chain. The conversion of these takes place in the usual way. [See also Pigulevsky (1928) and Subramaniam (1929).]

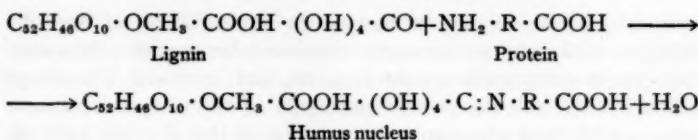
Up to now the nutrition of higher fungi has been studied very inadequately. This is explained by the impossibility of having at the disposal of investigators forest and field mushrooms of definite composition and origin. The cultivation of *Agaricus (Psalliota) campestris* on a large scale would require, so it would seem, knowledge of the physiology of this mushroom. Unfortunately its cultivation has been in the hands of market-gardeners who scarcely knew how to approach the problem from a scientific point of view. Only in the last few years has a series of important studies been carried out. Styer has shown that the mycelium of the field agaric grows on a variety of substances containing sugar, hemicellulose, cellulose, lignin, and protein. Hein (2, 3) and Lambert found that upon the compost there may be grown mushrooms suitable for the market. Waksman & McGrath studied the chemical transformations taking place in farmyard-manure during the growth of the fungi, and noted considerable changes in the composition of the micro-organisms. Moreover, it was observed during different stages of the decomposition of the manure that growth of the fungi was attended by a considerable decrease in the content of cellulose, lignin, and the water-soluble substances. Waksman & Nissen (1) have shown that the lignin and protein of the compost are the most essential nutritive substances for growth of the fungi. In a new work, Waksman & Nissen (2) studied the chemical transformations of manure composts, in different stages of their development, as well as when utilized for growing *Agaricus campestris*. Particularly interesting are the data of the authors on the chemical changes taking place in the constituent parts of fresh and of compost manure under the influence of the field agaric grown in pure culture in a sterilized substrate. The chemical changes in the medium were studied by the methods of Waksman & Stevens. Growth on composts was charac-

terized by a considerable decrease in the lignin content of the substrate, as well as by an increase in water-soluble organic substances, nitrogenous compounds soluble in water, and ammonia. The fungi utilized fairly well the protein and lignin of the compost. In connection with these observations it was proved that the best yield of fungi was obtained from composts which received an addition of 25 per cent (dry weight) of wheat straw, as well as the necessary amount of nitrogen and of nutritive mineral substances. The works of Waksman & Nissen supply new material for the scientific cultivation of fungi.

The important question concerning the decomposition of proteins by micro-organisms was studied by Waksman & Starkey from the point of view of their transformation by soil micro-organisms, in particular the soil fungi. In availing themselves for this purpose of albumin, edestin, gliadin, zein, and casein as proteins, and of the fungi, *Rhizopus*, *Trichoderma*, *Actinomyces*, and of a series of bacteria, the authors noted considerable differences in the degree of decomposition, between the plant and the animal proteins; differences were also noted between decomposition by bacteria and decomposition by fungi.

Continuing his study of the chemical nature and the origin of humus, Waksman, in collaboration with Iyer (1), obtained under laboratory conditions the "humus nucleus" and studied its influence on the microbiological processes of the soil. It turned out that the addition of lignin to proteins being decomposed by pure cultures of bacteria and fungi (*Actinomyces* and *Trichoderma*) considerably checked the decomposition of the proteins. This is not due to the toxic action of lignin on the micro-organisms, but is the result of compound formation between lignin and proteins, as such a combination has been shown to be quite highly resistant to decomposition by bacteria.

The authors synthesized this complex in the laboratory. By its physical, chemical, physico-chemical, and biological properties it proved to be akin to the organic soil substance usually termed as "humus" or "humic acid." The ligno-protein obtained binds different metals: Ca, Mg, Fe, Al, in the same way as the soil humus. The authors designate this ligno-protein as "humus nucleus." Taking into consideration the data of Fuchs on the chemistry of lignin, the authors suggest the following scheme for the combination of lignin with protein.



In a second work Waksman & Iyer (2) studied the influence of the synthesized "humus nucleus" on several important microbiological processes. Mention should be made of the decomposition of the mycelium of *Trichoderma* by micro-organisms in sand, in the absence and in the presence of lignin. Judging by the formation of CO_2 and the accumulation of NH_3 the lignin checked mycelial decomposition. It turned out that at the end of the process the nitrogen of the mycelium contained the ligno-protein complex. A similar influence was exerted by the lignin on the decomposition of casein. It should also be noted that the synthetic humus exerted a highly favorable influence on the fixation of nitrogen by *Azotobacter*.

ON NITROGENOUS SUBSTANCES, PIGMENTS, AND INORGANIC CONSTITUENTS

Mirande directed his attention to the fact that by means of micro-chemical tests applied to the lamellae of the fungus *Marasmius oreades*, HCN could be detected (but not in the mycelium or spores). The fruit bodies liberated HCN on cold-water extraction, but to a greater degree when boiled. In *Clitocybe geotropa*, HCN was also observed.

With regard to nitrogenous transformations in fungi it should be mentioned that Schober (1930) has conducted experiments concerning the fixation of atmospheric nitrogen by *Aspergillus niger*. The question has been repeatedly raised by investigators and has been solved occasionally in the affirmative, occasionally in the negative. The positive data of Schober, however, have found no confirmation in the new work of Mathilde Schröder. The latter author repeated the work of Schober with the same races and found no greater increase of nitrogen in the flasks than 0.1 mg., which may be explained by the adsorption of traces of bound nitrogen from the air during the prolonged experiment. Thus the faculty of mould fungi to fix nitrogen is once more called into question. Schröder tends to explain the positive results of Schober by the fact that the latter introduced into his cultures a certain catalyst, which remained

unnoticed by the author himself. The data of Schröder have been lately confirmed by Roberg, who tested twenty-one races of *Aspergillus*. Five of them had given positive results in the work of Schober. In spite of having tested different nutritive media, supplemented with various metals (Zn, Cu, Wo, Mo) as catalysts and though soil passage (*Erdpassage*) of the fungus was resorted to, Roberg could in no case detect nitrogen-fixation. In comparing these and other experiments concerned with the fixation of atmospheric nitrogen, one is inclined to seek an explanation of conflicting results in the variation of the physiological functions of the micro-organisms. It is possible that some races of *Aspergillus niger* are endowed with the faculty of fixing nitrogen, but this physiological character may easily be lost in artificial culture and cannot be restored. Our acquaintance with the loss of toxic properties, as well as with the fixation of nitrogen in bacteria, justifies this supposition. Also Kadelbach affirms that the cultures of which Schober availed himself for his experiments on nitrogen assimilation lost this faculty after having been grown on malt-agar. This fact, as well as the recognition that variations exist in cultures grown from one spore, points to the faculty of *Aspergillus* to undergo mutations.

Among the pigments of the fungi, Bergmann (1, 2) investigated two substances from ergot—"ergoflavine" and "ergochrisine." In extracting alkaloids from ergot there had been isolated long ago crystalline yellow pigments described by different authors under different names. Freeborn (1912) described one of them as "ergoflavine," of the composition $C_{15}H_{14}O_7$, referring it to the flavones. Bergmann, by acetylation of ergoflavine, obtained a penta-acetate, thus demonstrating the presence of 5 hydroxyl groups. He referred it to vitexin. On boiling ergoflavine with weak alkali, water is added and one obtains ergoflavonic acid, $C_{15}H_{16}O_8$, possessing a lactone ring. In consequence the function of two non-hydroxyl atoms of oxygen is established. The second of the above-mentioned pigments, discovered in ergot by Jakobi (1897), was called by him ergochrisine with the formula $C_{21}H_{22}O_9$. Bergmann obtained it from chloroform solution in the form of beautiful golden-yellow leaflets. Kraft (1906) gave it the formula $C_{14}H_{14}O_6$, but according to Bergmann the composition of ergochrisine is $C_{28}H_{28}O_{12}$. On acetylation ergochrisine-decaproacetate, $C_{48}H_{48}O_{22}$, was obtained. On fusion with alkali the following breakdown-products were obtained: oxalic acid, acetic acid, evidently also 1-carboxylic 3-methyl 5-hydroxy cresotinic acid,

$\text{HOOC}\cdot\text{C}_6\text{H}_3\cdot(\text{CH}_2)\text{OH}$; from the phenol fraction, resorcin and a second phenol, tetra-hydroxy-diphenyl, were isolated. It is interesting to note that up to the present time derivatives of diphenyl have been rarely met with among plant substances.

Nishikawa isolated from the mycelium of *Monascus purpureus* W. two pigments—monascorubrin ($\text{C}_{22}\text{H}_{24}\text{O}_5$) in red prisms or acicles, and monascoflavin ($\text{C}_{17}\text{H}_{22}\text{O}_4$), in rhombic yellow plates; when melted with alkali, monascorubrin produces a small amount of fatty acids, chiefly capronic acid, $\text{CH}_3(\text{CH}_2)_4\text{COOH}$. The pigment contains one double bond, a straight chain of six carbon atoms, and, presumably, a benzoyl nucleus.

As regards the ash elements of the fungi, the data of Rippel & Stoess concerning the calcium requirements of mould fungi should be mentioned. To speak of a general requirement of the fungi for calcium would be erroneous, as usually the fungi, among them *Aspergillus niger*, develop quite normally without it. However, this element in the fungi may play a certain rôle, not merely as a catalyst. The fungi *Rhizopus*, *Penicillium*, *Fusarium* are not indifferent to it. According to the authors, the influence of calcium shows itself in its antagonistic action towards magnesium.

According to the data of Mme Levi, aluminium is not indispensable to *Sterigmatocystis nigra*, as in cultures entirely deprived of this element the fungus develops normally.

Manceau, and Bretin, Manceau & Rey have studied the influence of different salts (magnesium chloride, copper acetate, nickel, and cobalt) on the metabolism of *Penicillium glaucum*. MgCl_2 favors the combustion of sugar, but does not influence the accumulation of phytosterol.

The influence of ZnSO_4 on the transformation of substances in *Aspergillus niger* has again been studied by Porges, who showed that zinc induces considerable changes in the composition of the fungus, in the amount of ether-soluble and water-soluble substances, and in hemicelluloses; moreover, the accumulation of citric acid increases.

Mention should be made of the interesting work of Mousseron & Fauroux on the concentration and rôle of zinc in higher fungi. Starting from the work of Delezenne (1919), who established the fact that zinc is an element characteristic of snake poison, the authors attempted to find a connection between the zinc content of the higher fungi and their hemolytic and nucleolytic properties. In the

latter case the triturated mass of the fungus was allowed to act on nucleinate; the nucleolytic force was estimated by the amount of phosphate liberated. In the table given by the authors the hemolytic and nucleolytic faculties are indeed parallel to the zinc content of the fungi. An exception is the fungus *Hygrophorus conicus*, which contains much zinc and possesses no hemolytic activity but instead possesses significant power of agglutination.

Fungi	Content of Zinc in mg. per kg.		Hemolytic Activity	Nucleolytic Activity
	Fresh Substance	Dry Substance		
<i>Clavaria aurea</i>	3.9	41	—	0.53
<i>Morchella esculenta</i>	64.1	—	0.83
<i>Hydnium imbricatum</i>	5.89	67	—	0.55
<i>Hydnium vitum</i>	6.16	69	—	0.13
<i>Boletus edulis</i>	8.20	74	—	0.93
<i>Lactarius deliciosus</i>	3.93	86	—	0.40
<i>Boletus granulatus</i>	7.80	88	—	0.80
<i>Claviceps purpurea</i>	90	—
<i>Amanita avoidea</i>	6.46	95	—	0.43
<i>Tricholoma sulfureum</i>	7.34	124	+	2.93
<i>Hygrophorus conicus</i>	10.92	136.6	— (agglutinin)	0.40
<i>Amanita muscaria</i>	10.20	167	+	3.13
<i>Amanita verna</i>	11.36	186	+	1.93
<i>Amanita pantherina</i>	16.83	202	+	1.76
<i>Russula emetica</i>	42.20	211	+	1.80
<i>Helvelia crispa</i>	34.06	262	+	2.26
<i>Pleurotus olearius</i>	45.35	279	+	2.73

ON THE CHEMICAL COMPOSITION OF THE HIGHER FUNGI

Among the recent works on the chemical composition of the higher fungi mention should be made of the analyses carried out by Saburow & Wasiliew in regard to fifteen mushroom species used for food in Moscow. Great differences in protein content were observed. Thus:

	Protein in Percentage of Dry Substance
<i>Agaricus campestris</i>	32.06
<i>Boletus edulis</i>	31.25
<i>Tricholoma portentosum</i>	10.50
<i>Collybia velutipes</i>	8.87

The fluctuations in the fat content were also found to be considerable: in *Boletus edulis*, 1.6 per cent; in *Boletus scaber*, 9.69 per cent.

Recently, Sabalitschka, from former analyses of mushrooms, once more raised the question as to their value as a foodstuff. He is inclined to recognize that from the amount of nitrogenous substances, fat, and carbohydrates, fresh mushrooms may rank with fresh vegetables as regards their nutritive value. However, even from the data of Sabalitschka, it may be seen that some of the mushrooms (*Agaricus campestris*, *Boletus edulis*) are of an unusually high protein content. Therefore no conclusions may be drawn on the basis of any average analytical data. It will be necessary to obtain new data based on exact modern methods. The question as to the utilization of mushrooms, in particular their protein, for nutritive purposes remains unsettled.

ON GROWTH-PROMOTING FACTORS, VITAMINS, AND ENZYMES

As has been established by recent investigations the growth of plants is stimulated by substances termed regulators of growth. Some of these substances accelerate cell-division (Haberlandt's *Wundhormone*, 1922), while others influence the growth of the plant in length. The best object on which to test the action of these regulators is the coleoptile of oats. In 1930 Nielsen (1) studied the action of the substance produced by the fungus *Rhizopus stiiinus*, called by him rhizopin. Up to now rhizopin could not be obtained in pure condition, but has been purified so far that 1/60,000 mg. suffices for calling forth a reaction in the coleoptiles of oats and other plants. Nielsen showed further that in augmenting the dose of rhizopin the growth of *Aspergillus niger* may be accelerated, but afterwards the growth slackens. Finally, in a new work, the author mentions a substance from *Boletus edulis* which accelerates growth. This substance, isolated from desiccated fungi, resembles rhizopin by its action on the coleoptiles of oats and by its solubility in 87 per cent alcohol. It is not resistant to oxygen, and when shaken with H_2O_2 breaks down. In the common *Agaricus campestris* this substance has not been found.

Parallel with the investigations of Nielsen, work was performed in this field by Boysen-Jensen (1), who demonstrated that, in cultivating *Aspergillus niger* in solutions of peptone or hemoglobin, a great number of regulators of growth are formed. In a later work, (2), the author tested the influence of a series of amino-acids on the formation of regulators of growth. Tyrosine turned out to be the best of the cyclic amino-acids, leucine and lysine of the aliphatic ones; the other amino-acids with the exception of tryptophane were

of no importance. It is interesting to note that the substance extracted from *Aspergillus niger* seems to be of no physiological importance for the fungus itself.

Mention should be made of the very important data of Euler and Euler & Philipson, associating the substances of growth from mould fungi and the mould "bios" of yeast. It was proved that in the cultures of *Rhizopus tritici*, *Rhizopus nigricans*, *Aspergillus Wentii*, and *Penicillium* substances were synthesized, which were actual growth factors (*Zuwachs faktor*) for yeast.

From this it may be seen that a series of substances bearing the names "bios," "rhizopin," "growth regulators," etc., may be associated with regard to their action. But up to the present we have no definite data that would give us the right to identify them chemically as well as to consider them part of the vitamin-B complex.

There is an ever-increasing number of instances in biochemical literature which bring us to the conclusion that living cells are very sensitive to the admixtures contained in our so-called chemically pure preparations. This may be illustrated by the recent works of Schopfer (1, 2), who studied the causes of zygote formation in *Phycomyces Blakesleeanus*. It was established that this fungus develops better and forms more zygotes on maltose from Kahlbaum than on preparations of chemically pure maltose from Schuchardt or Merck. In order to solve the question of growth and zygote formation in a nutritive medium containing pure maltose, the author added either dry yeast, or extracts of beer yeast, or 0.0025 to 0.005 gm. of vitamin B prepared according to Harris. The results were as follows: in the control media the zygotes were not numerous (thirty-five), on yeast there were 1,000, in water-extract from yeast 900 to 950, in alcohol extract 1,200 to 1,400, while in the vitamin preparation of Harris the zygotes were innumerable and the mycelium developed well. Schopfer holds that the active substance in growth and zygote formation is the thermostable vitamin B. Burgeff is of the opinion that there exists a special sexual substance penetrating from without through the thin membranes and stimulating the sexual reaction of the opposite mycelium, in result of which a zygote is formed. Ronsdorf tried to corroborate this hypothesis, in testing the influence of the sap of different races of a fungus on one another, but obtained no sexual reaction. Testing a series of substances which might induce the formation of zygotes, the author demonstrated that histamine, in very small doses, promotes zygote

formation. The author is inclined to think that this process is conditioned by a substance closely related to histamine. However, keeping in view the above-mentioned data of Schopfer, the question naturally arises as to whether vitamin B was not admixed with the histamine, thus determining zygote formation.

With regard to the vitamins of higher fungi, it is known from the data of Scheunert & Reschke that of all the fungi the richest source of vitamin A is the yellow *Cantharellus cibarius*. With the usual method of cooking, as well as of sterilization, the vitamin A remains undestroyed. Vitamin B was observed in the series of fungi investigated, only in small amount, but the content of vitamin D was high.

Much interest regarding the antirachitic vitamin of fungi has been aroused by work of Pruess *et al.* These investigations pertain to the sterol content of fungi living under natural conditions, as well as that of 55 moulds grown in a synthetic nutritive medium containing 4 per cent of glucose. The mould fungi grown under uniform conditions produced different amounts of sterols:

<i>Aspergillus oryzae</i> (965)	0.98 per cent
<i>Penicillium janthivellum</i>	0.16 per cent
<i>Penicillium expansum</i>	0.35 per cent
<i>Aspergillus niger</i>	0.4 per cent

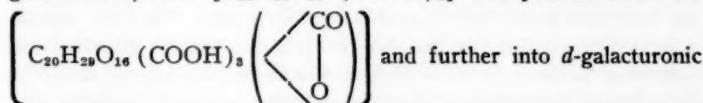
The sterol content depends not only on the species but also on the strain of the fungus; under uniform conditions of cultivation four different strains of *Aspergillus oryzae* produced 0.76, 0.63, 0.54, and 0.98 per cent of sterol. The period of cultivation of the fungus also exerts an influence on the accumulation of sterol. Thus, after 10 days, *Aspergillus oryzae* produced 0.63 per cent of sterol, and after 47 to 51 days, 1.07 per cent. Besides, the accumulation of sterol depends on the conditions of cultivation, and on the yield of mycelium.

The dried, autoclaved, and finely ground material of moulds and fungi was exposed to ultra-violet rays and afterwards administered to rachitic rats in daily doses of 10 mg. Three higher fungi, *Marasmius oreades*, *Hypholoma incertum*, and *Secotium acuminatum*, as well as eight moulds, *Aspergillus niger*, *Aspergillus oryzae*, *Rhizopus niger*, etc., exerted an antirachitic action.

In their further work, Pruess, Gorcica, Greene & Peterson extended their researches in regard to the separate species of fungi.

They investigated 30 species of *Aspergillus*, 20 of *Penicillium*, and 15 species of other moulds and showed that the nature of the medium is of great importance for the accumulation of sterols; conditions favoring growth promoted the synthesis of sterols. The sterol content of the fruit bodies of fungi grown under natural conditions is usually lower than in *Aspergillus* grown on an artificial medium.

Among the works pertaining to the enzymes of fungi those of Ehrlich (1, 2) on the new enzyme, pectolase, discovered by him, should be mentioned. This enzyme is able to split pectolic (tetragalacturonic) acid $[C_{20}H_{28}O_{17} (COOH)_4]$ into pectolactonic acid



acid. The author used as a source of the enzyme a crude preparation of taka-diastase from the moulds *Penicillium glaucum*, *Penicillium Ehrlichii*, as well as the purified enzyme isolated from these preparations. Particularly active pectolase was observed in *Penicillium Ehrlichii*.

The enzymes of *Aspergillus carbonarias* have been studied by the Spanish investigator Bustinza. According to his data this fungus forms invertase, catalase, maltase, amylase, and emulsin. Purified preparations of invertase and amylase were obtained; in the latter case the enzyme was accumulated by absorption by raw potato starch.

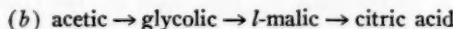
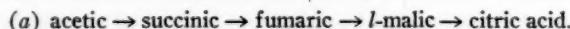
ON THE ORGANIC ACIDS OF FUNGI

During recent times the formation of organic acids by fungi has been intensively studied. At the present moment it is widely accepted that citric acid is formed from the products of glucose-splitting. Even in 1909 Euler recognized the importance of acetic aldehyde, which may condense and not only produce malic, tartaric, succinic, and fumaric acids, but also citric acid and aconitic acid. Bauer (1913) expressed the view that malic and citric acids are formed as condensation products of glycolic acid.

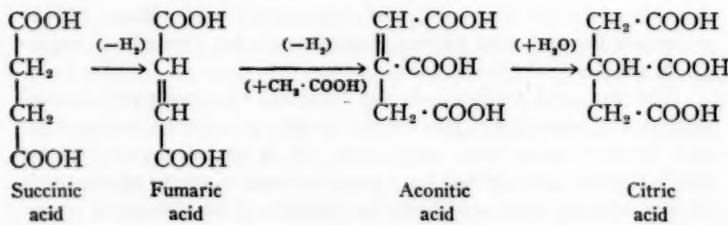
The works of Butkewitsch and Fedoroff have positively demonstrated that some fungi possess the faculty of synthesizing succinic and fumaric acids from acetic acid. It is to Bernhauer and his pupils that we are indebted for a great number of works on the mode of formation of citric acid from the products of breakdown of sugar.

In investigating 28 strains of *Aspergillus niger*, the authors were able to prove that, during the formation of citric acid from sugar, as an intermediate or by-product, malic acid was obtained. From 6.5 liters of culture liquid containing 975 gm. of cane sugar, 2 gm. of malic acid, along with 500 gm. of citric acid, were obtained. The presence of malic acid is additional proof of the theory of citric-acid formation from acetic acid, through succinic and fumaric acids.

It must be noted that, according to Bernhauer & Scheuer, a series of strains of *Aspergillus niger* are able to produce from acetates, glyoxylic and glycolic acids, which in turn may be converted directly not only into oxalic acid, but also into citric acid. Thus the formation of citric acid is also possible through the stage of glycolic acid, according to the scheme of Bauer. Chrzaszcz and his collaborators investigated five races of *Penicillium* cultivated in a medium with 50 cc. of ethyl alcohol. As an intermediate product acetic acid was always obtained; later, citric acid formed. In three races, fumaric acid and *l*-malic acid accumulated; in two, glycolic acid, and in one, succinic acid and oxalic acid. Thus mould fungi build up from alcohol the same substances as from sugar. In this connection the authors have drawn up two schemes for the formation of citric acid from ethyl alcohol:

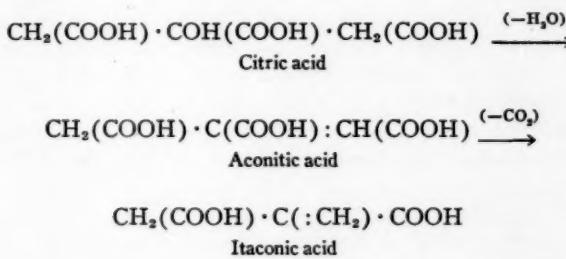


Bernhauer & Böckl (1) have shown that citric acid may be obtained from alcohol in yields up to 25 per cent of the theoretical amount. At the same time the formation of oxalic acid as well as of malic and tartaric acids may be observed. In addition to these schemes for the formation of citric acid, another pathway, through aconitic acid, should be mentioned.



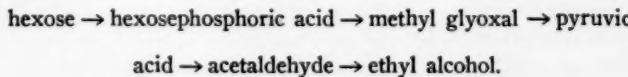
The possibility of such a scheme has been pointed out by Butkewitsch. Experimental proofs were given by Bernhauer & Böckl (2), who showed that in growing the fungus on 2.4 per cent potassium aconite there was obtained 23.2 to 25.8 per cent of citric acid; conversely, with cultivation on citric acid a qualitative reaction for aconitic acid was obtained.

The connection between aconitic and citric acid finds still stronger confirmation in the discovery by Kinoshita of itaconic acid, HOOC·CH₂·C(:CH₂)·COOH. This acid was the product of metabolism of *Aspergillus itaconicus*, found under natural conditions in the expressed sap of pickled plums. In culture the fungus develops on 20 per cent sugar solution. If a lower concentration is used, common salt or sodium sulphate are added, in order to increase the osmotic pressure. In such a medium an unsaturated dibasic acid, of the elementary composition C₅H₆O₄, was accumulated which, according to Kinoshita, was itaconic acid. *Aspergillus itaconicus* may also utilize citric acid and, when cultivated on sugar with calcium carbonate, citric acid may be obtained. All this permits the following scheme for acid formation:



The authors did not observe aconitic acid as an intermediate product, but found that itaconic acid accumulated first and afterwards disappeared.

For the early stages of the transformation of sugars into acids by mould fungi, Chrzaszcz, Tiukow & Zakomorný have established the following course of reactions:



Recently Suthers & Walker have demonstrated along these lines that, as yeast (according to Neuberg & Kobel) forms methyl glyoxal from hexosephosphoric acid, so this substance may be obtained by means of *Aspergillus niger*, in the presence of toluol. The new data of Bernhauer & Thelen show that in the formation of organic acids from sugar, it is possible by means of sodium sulphite to obtain acetaldehyde up to 60 per cent of the theoretical quantity. A new proof according to Bernhauer: "dass auch bei Säurebildung durch Pilze aus Zucker der Weg über die durch das Neuberg'sche Abbau-schema gekennzeichneten Stufen führen dürfte."

Among the researches endeavoring to throw light on the conditions which attend the formation of citric acid, the investigation of Frey should be mentioned. It shows that the different fungi require a different optimum pH for the formation of citric acid; for *Aspergillus niger* the optimum pH is 2, while *Citromyces glaber*, which produces no citric acid under these conditions, requires pH 3 to 4.

The mutual relation between organism and medium in *Aspergillus niger* has been studied more deeply, from the point of view of the oxidation potential, by Kusnetzow (in the laboratory of E. E. Uspensky). The author showed that on the fourth day of cultivation, when the rH of the medium was 18.5, no citric acid could be observed, while on the seventh day, with rH = 15.5, citric acid appeared. The cultivation of the fungus in an atmosphere of hydrogen led to a decrease in rH from 13.7 to 3.9 and 1.4. In this case no citric acid was formed, for in the strongly reducing medium the intermediate products of sugar metabolism could not be oxidized to citric acid.

With regard to the development and the measurement of growth energy in *Aspergillus niger* two very valuable works, those of Terroine & Wurmser and of Algera, have been published.

Gluconic acid is a valuable product for medicinal purposes. In this connection the work (unpublished) of E. K. Kardo-Ssysojewa, of the Leningrad Institute for Food Investigation, on the production of gluconic acid by means of *Aspergillus niger* is of interest. Kardo-Ssysojewa cultivated *Aspergillus niger* on the usual nutritive solution of sugar and salts, whereby the latter were diluted 5, 10, and 25 times. The best results were obtained when the concentration of the salts was 25 times weaker, and the experiments were conducted in the presence of calcium carbonate. Good yields were obtained when the solution on which the mycelium had grown was poured off, and a

new solution of sugar was added. At a temperature of 29° to 30° C., after four or five days of growth, in the presence of 12 gm. of CaCO_3 the following results were obtained:

Time	Sugar Consumed	Gluconic Acid Formed
4 days.....	11.36 gm.	11.89 gm.
5 days.....	11.76 gm.	12.19 gm.

i.e., the yield of gluconic acid is almost 100 per cent; no oxalic acid and very little citric acid were formed.

With regard to the relation of the acids to each other, it must be noted that Hofmann has investigated the decomposition of *d*-glucuronic and *d*-galacturonic acids by the fungus *Aspergillus niger*; in both cases a considerable amount of oxalic acid was accumulated, though citric acid was not observed.

Raistrick (1931) has shown that the formation of kojic acid, $\text{C}_8\text{H}_8\text{O}_4$ (5-hydroxy 2-hydroxymethyl γ -pyrone), discovered by Saito (1907) may serve as a diagnostic character for the *Aspergillus* species belonging to group A (*flavus, oryzae, tamarii*) and that this acid is formed not directly from sugar, but from the products of its breakdown, since the fungus can build it up from combinations containing not only 6, but even 5, 4, or 3 atoms of carbon. In his recent work on *Aspergillus oryzae* Tamiya has shown that this fungus utilizes also carbohydrates (hexose, mannose, fructose) for the formation of kojic acid as well as pentoses (arabinose, xylose), polyatomic alcohols (glycerol, erythritol, adonitol, mannitol, sorbitol, dulcitol, inositol, etc.) and some acids (succinic acid).

May and his collaborators have shown that among the substances stimulating the formation of this acid in *Aspergillus flavus* there is ethylene chlorhydrin ($\text{CH}_2\text{OH} - \text{CH}_2\text{Cl}$). In order to increase the yield of kojic acid it suffices to add 100 mg. of ethylene chlorhydrin to one liter of the culture medium.

Different races of mould fungi supply new acids. Birkinshaw & Raistrick have just published data showing that *Penicillium puberulum*, which induces the decay of corn, produces besides penicillic acid [discovered by Alsberg & Black (1913)], two other acids, namely, puberulic acid ($\text{C}_8\text{H}_8\text{O}_6$) and one of the empirical composition $\text{C}_8\text{H}_4\text{O}_6$. The isolation of these acids was based on the formation of insoluble red precipitates with nickel sulphate. Birkinshaw & Raistrick are of the opinion that these acids may play a rôle in the oxi-

dative-reducing systems of the organism. Penicillic acid ($C_8H_{10}O_4$), when titrated with phenolphthalein, appears as a monobasic acid, its structure being unknown. It has toxic properties, 0.2 to 0.3 gm. per kilo being the minimum lethal dose for the animals investigated.

In concluding this survey we wish to note the progress in our understanding of the processes of organic-acid formation in fungi. The knowledge of these processes will certainly be made use of in all other fields of plant chemistry. Thus the genesis of organic acids in fruit will be illuminated. Owing to the exact studies on the origin of organic acids, it will be possible to improve considerably the technique of acid production by means of fungi. The determination of the most productive strains of fungi must be considered as a very important factor. Nor will it be sufficient simply to find suitable races; we shall have to learn how to re-activate them when they have suffered in the course of time a loss of their acid-forming properties. Much effort will be required before we succeed in securing an uninterrupted production of citric and other acids by the mould fungi.

As before, much is expected from fungi (and yeast) as sources of vitamins B and D. New and valuable results are anticipated in this domain.

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